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The Journal of Biochemistry, Vol. 47, No. 6, 1960

CHROMATOGRAPHIC STUDIES ON BACTERIAL FATTY ACIDS*

By KUNIHIKO SAITO**

(*From the Department of Biochemistry, Faculty of Medicine, Nagoya City University, Nagoya*)

(Received for publication, August 20, 1958)

Using reversed-phase column chromatography, the fatty acids composition of acetone soluble lipids derived from 5 kinds of bacterium were analysed.

EXPERIMENTALS AND RESULTS

Analytical Method—In general the methods of Howard and Martin (*1*) and Silk and Hahn (*2*) were followed. The column dimensions were 1×100 cm. and the rate of flow was kept at 40~50 ml. per hour. As a preliminary test, model experiment was performed by combining the methods employed by Crombie *et al.* (*3*), Yamakawa *et al.* (*4*), etc. Firstly a mixture of authentic fatty acids was made, which was supposed to be favourably compared with the present bacterial fatty acids; *viz.* the mixture contained myristic (50 mg. or 22 per cent), palmitic (75 mg. or 30 per cent), oleic (95 mg. or 34 per cent) and linoleic (40 mg. or 14 per cent) acids, making the total 260 mg. One hundred mg. of the mixture were oxidized by the Bertram method and another one hydrogenated by using the platinum oxide. These two kinds of treated samples and the original mixture for the model experiment were subjected to chromatography. The oxidation and the hydrogenation were performed in the following manner.

Oxidation; according to Crombie *et al.* (*3*), 100 mg. of the original mixture were dissolved in 1 ml. of 1/2 N methanolic potassium hydroxide and 5 ml. of water and methanol was evaporated by heating. After cooling 0.2 ml. of 50 per cent potassium hydroxide and 30 ml. of 5 per cent potassium permanganate were added gradually under constant shaking. The oxidation was allowed to proceed for 18 hours at 18°, after which 2 ml. of 50 per cent sulfuric acid were added, and the mixture was decolorized with saturated sodium hydrosulfite solution. Then it was heated up just to simmer, and after cooling it was extracted with petroleum ether. The extract was washed with 5 per cent aqueous solution of sodium bicarbonate and water, then petroleum ether was removed under reduced pressure; finally it yielded 60 mg. (*i.e.* 60 per cent of the material used) of oxidized white substance, the iodine value being zero.

Hydrogenation; 100 mg. of the original mixture dissolved in 10 ml. of absolute ethanol were shaken together with 10 mg. of freshly prepared platinum oxide in hydrogen atmosphere for about 40 minutes. After removing the catalyst and the solvent, 101 mg. of saturated white crystalline substance were obtained, the iodine value being zero.

The chromatograms and the numerical data are seen in Fig. 1 and Table I.

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* This work was read at the 4th Symposium on Bacterial Toxin held in Hakone, August 9th, 1957.

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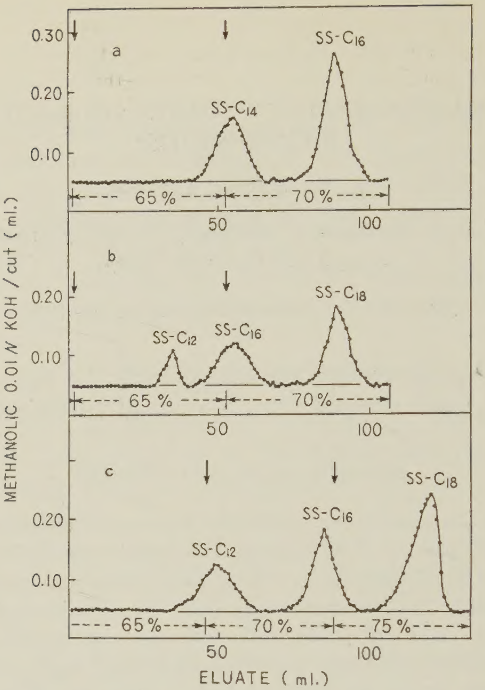

FIG. 1. Chromatographic patterns in three forms of a synthetic mixture consisting of myristic, palmitic, oleic and linoleic acids; a. Non-treated sample, b. Oxidized sample, c. Hydrogenated sample. About 10 mg. of each sample was chromatographed and the recovery was more than 96 per cent. Changes in developing solvents were indicated by arrows.

TABLE I

Result of Chromatographic Analysis of a Synthetic Mixture Including Myristic, Palmitic, Oleic and Linoleic Acids

The Name of Peak	Fatty Acids Analyzed			Unsat'd Acids (IV)%	Reduced Acids by Hydrogenation (V)%	Fatty Acids Found (VI)%
	Non-treated (I)%	Oxidized (II)%	Hydrogenated (III)%			
Lewer		8				
$SS\text{-}C_{12}$						
$SS\text{-}C_{14}$	38	23 (myristic)	23 (myristic)	15 (I–II)		myristic 23 linoleic 15
$SS\text{-}C_{16}$	62	29 (palmitic)	30 (palmitic)	33 (I–II)		palmitic 29 oleic 33
$SS\text{-}C_{18}$			48 (stearic)		48 (III–II)	
	100	60	101	48	48	

From the last column of the table it may be assumed that the chromatographical findings agree quite satisfactorily with the synthetic recipe. The details of this deduction are as follows: in Fig. 1-b, and consequently in Table I, oxidized sample (II), all those should be saturated acids, so that the peaks ss-C_{14} and ss-C_{16} seen in the chromatogram must be due to myristic and palmitic acids respectively—the one on the left not being obserbed in the case of the non-treated sample (see Fig. 1-a) and corresponding to ss-C_{12} is the oxidation products. (Note: the abbreviation ss-C_{14}, is the symbol denoting a peak in chromatogram including all the fatty acids which can be eluted together with saturated straight chain C_{14} acid.) The three saturated acids were found in molar ratio by 8, 23 and 29 per cent, totaling 60 per cent. The difference between non-treated sample (I) and oxidized one (II), *i.e.* 48 per cent (15 per cent for ss-C_{14} and 33 per cent for ss-C_{16}) is the part lost from the unsaturated acids by the oxidation.

The hydrogenation produced, as seen from Fig. 1-c or the (III) column of Table I, only ss-C_{18}, *i.e.* stearic acid, by the percentage of 48; in other words, the unsaturated acids coexisted in either ss-C_{14} or ss-C_{16} were those of C_{18} acids, or more precisely that vanished 15 per cent from ss-C_{14} was linoleic acid and the 33 per cent from ss-C_{16} was oleic acid. Thus the final results of the analysis would be those shown together in the last column of Table I, and as has been stated they are in good accordance with the initial composition of the mixture.

By using the above technique, it is possible to fractionate 200 to 300 mg. of a mixture of bacterial fatty acids into the individual constituents respectively.

Preparation of Bacterial Lipids—The bacterial species used are: (1) *B. alcaligenes faecalis*, (2) *S. pullorum*, (3) *B. fluorescens*, (4) *S. typhi murium* and (5) *B. Natto.* The bacterial cells were grown on ordinary broth for 5 days, and the moist bacteria harvested were dried *in vacuo.* The dried material was thoroughly pulverized in a stainless steel ball mill and extracted three times successively with ether-ethanol (1 : 1) at room temperature. After washing the extract with water and removed the solvent, there remained dark brown greasy mass, corresponding to about 3 per cent on the dry weight of bacteria.

Fractionation of the Lipids into Acetone Soluble and Insoluble Parts—This was carried out by the usual ether-acetone method, *viz.* dissolution of the sample in as small amount of ether as possible and precipitation with four volumes of acetone under cooling with ice.

Separation of Fatty Acids from the Acetone Soluble Lipids—The acetone soluble fraction of these 5 bacteria, except *B. alcaligenes facelis*, was saponified with *N* methanolic potassium hydroxide for 6 hours and the unsaponifiable matter

TABLE II

Physical and Chemical Properties of the Bacterial Fatty Acids

	Color & State	Melting Point (°C)	Neutralization Value	Iodine Value
B. alcalibenes faecalis	yellowish brown semisolid	45~46	189.7	54.6
S. pullorum	yellowish semisolid	39 ~41	206.0	43.3
B. fluorescens	yellowish brown semisolid	37.5~40	210.3	48.6
S. typhi murium	yellowish semisolid	38 ~39.5	212.5	40.3
B. Natto	browon liquid	21.5~24.5	225.5	24.8

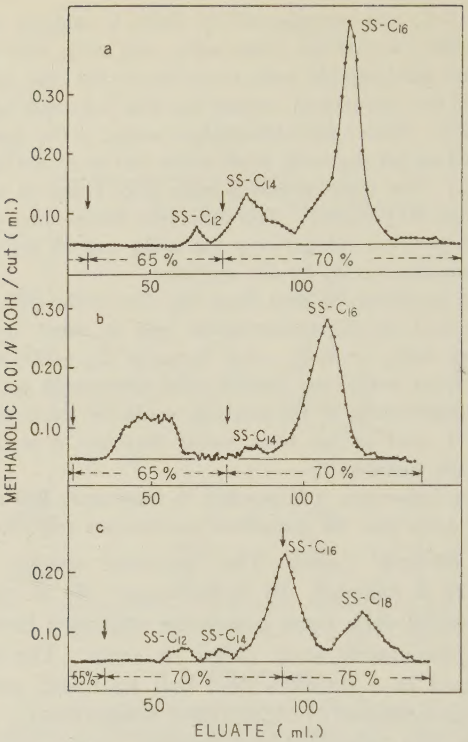

FIG. 2. Chromatographic patterns in three forms of the mixed fatty acids of *B. alcaligenes faecalis*: a. Non-treated sample, b. Oxidized sample, c. Hydrogenated sample. About 10 mg. of each sample was chromatographed and the recovery was more than 98 per cent.

TABLE III

Result of Chromatographic Analysis of the Fatty Acids of B. alcaligenes faecalis

The Name of Peak	Fatty Acids Analyzed			Unsat'd Acids (IV)%	Reduced Acids by Hydrogenation (V)%	Fatty Acids Found (VI)%
	Non-treated (I)%	Oxidized (II)%	Hydrogenated (III)%			
Lower		20				
SS-C_{12}	3		3 (lauric)			lauric 3
SS-C_{14}	22	3 (myristic)	3	19 (I–II)		myristic 3 unsaturated C_{16} & C_{18} 19
SS-C_{16}	72	43 (palmitic)	64	29 (I–II)	21 (III–II)	palmitic 43 unsaturated C_{16} & C_{18} 29
SS-C_{18}	3	2	30 (stearic)		28 (III–II)	
	100	68	100	48	49	

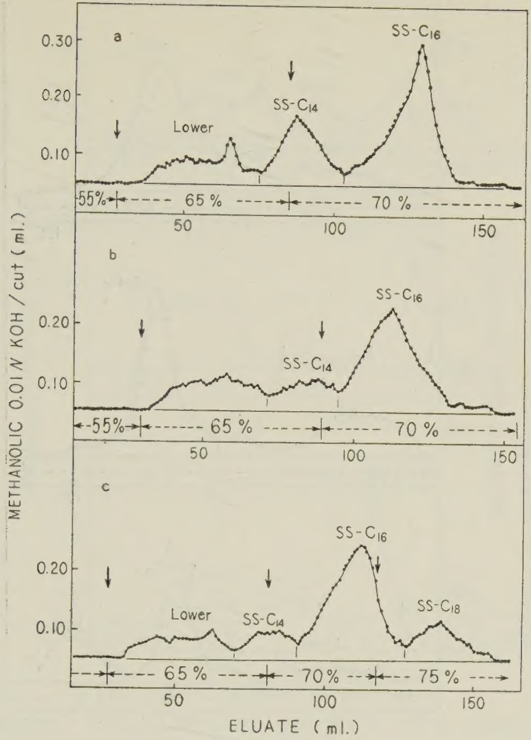

FIG. 3. Chromatographic patterns in three forms of the mixed fatty acids of *S. pullorum*: a. Non-treated sample, b. Oxidized sample, c. Hydrogenated sample. About 10 mg. of each sample was used and the recovery was more than 98 per cent.

TABLE IV

Result of Chromatographic Analysis of the Fatty Acids of S. pullorum

The Name of Peak	Fatty Acids Analyzed			Unsat's Acids (IV)%	Reduced Acids by Hydrogenation (V)%	Fatty Acids Found (VI)%
	Non-treated (I)%	Oxidized (II)%	Hydrogenated (III)%			
Lower	16	22	17			saturated C_{10}—C_{12} 17
SS-C_{12}						
SS-C_{14}	28	12 (myristic)	12	16 (I-II)		myristic 12 unsaturated C_{16} & C_{18} 16
SS-C_{16}	54	47 (palmitic)	56	7 (I-II)	9 (III-II)	palmitic 47 unsaturated C_{16} & C_{18} 7
SS-C_{18}	2	2	15 (stearic)		13 (III-II)	
	100	83	100	23	22	

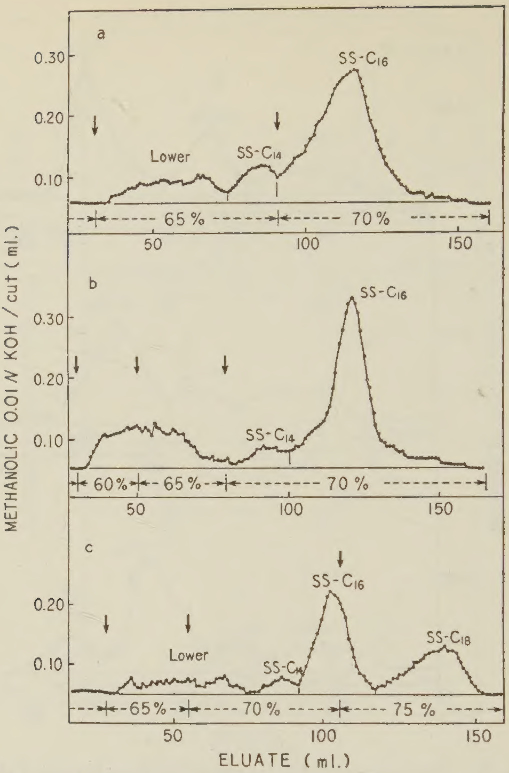

FIG. 4. Chromatographic patterns in three forms of the mixed fatty acids of *B. fluorescens*: a. Non-treated sample, b. Oxidized sample, c. Hydrogenated sample. About 10 mg. of each sample was used. Recovery was more than 98 per cent.

TABLE V

Result of Chromatographic Analysis of the Fatty Acids of B. fluorescens

The Name of Peak	Fatty Acids Analyzed			Unsat's Acids (IV)%	Reduced Acids by Hydrogenation (V)%	Fatty Acids Found (VI)%
	Non-treated (I)%	Oxidized (II)%	Hydrogenated (III)%			
Lower SS-C_{12}	17	16	15	2 (I-III)		saturated C_{10}—C_{12} 15
SS-C_{14}	10	4 (myristic)	3	6 (I-II)		myristic 4 unsaturated C_{16} & C_{18} 6
SS-C_{16}	70	31 (palmitic)	50	39 (I-II)	19 (III-II)	palmitic 31 unsaturated C_{16} & C_{18} 39
SS-C_{18}	3	2	33 (stearic)	1 (I-II)	31 (III-II)	
	100	53	101	48	50	

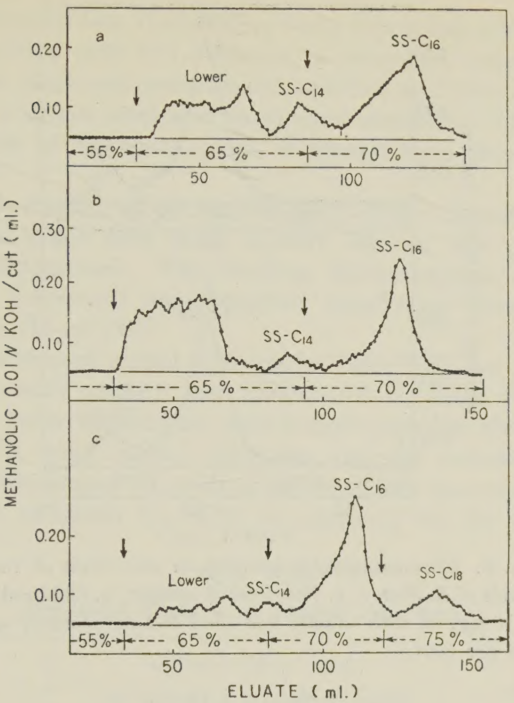

FIG. 5. Chromatographic patterns in three forms of the mixed fatty acids of *S. typhi murium*: a. Non-treated sample, b. Oxidized sample, c. Hydrogenated sample. About 10 mg. of each sample was used and the recovery was more than 98 per cent.

TABLE VI

Result of Chromatographic Analysis of the Fatty Acids of S. typhi murium

The Name of Peak	Fatty Acids Analyzed			Unsat'd Acids (IV)%	Reduced Acids by Hydrogenation (V)%	Fatty Acids Found (VI)%
	Non-treated (I)%	Oxidized (II)%	Hydrogenated (III)%			
Lower SS-C_{12}	37	48	18	19 (I–III)		saturated C_{10}—C_{12} 18 unsaturated C_{16} & C_{18} 19
SS-C_{14}	14	7 (myristic)	8	7 (I–II)		myristic 7 unsaturated C_{16} & C_{18} 7
SS-C_{16}	49	28 (palmitic)	57	21 (I–II)	29 (III–II)	palmitic 28 unsaturated C_{16} & C_{18} 21
SS-C_{18}			19 (stearic)		19 (III–II)	
	100	83	102	47	48	

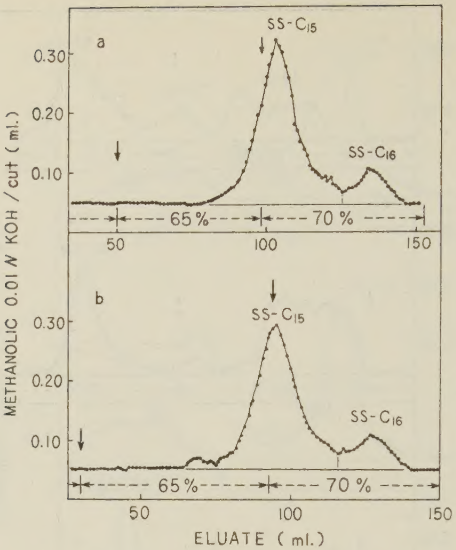

FIG. 6. Chromatographic patterns in two forms of the mixed fatty acids of *B. Natto*: a. Non-treated sample, b. Oxidized sample. About 10 mg. of each sample was used and the recovery was more than 98 per cent.

TABLE VII

Result of Chromatographic Analysis of the Fatty Acids of B. Natto

The Name of Peak	Fatty Acids Analyzed			Unsat'd Acids (IV)%	Reduced Acids by Hydrogenation (V)%	Fatty Acids Found (VI)%
	Nontreated (I)%	Oxidized (II)%	Hydrogenated (III)%			
Lower	0	3	—			
SS-C_{12}						
SS-C_{14}	0	0	—			
SS-C_{15}	88	74	—	14 (I-II)		saturated C_{15} acid 75 unsat'd Acid 14
SS-C_{16}	12	11 (palmitic)	—	1		palmitic
	100	88	—	15		

was removed and yielded a mixture of free fatty acids. In *B. alcaligenes faecalis*, however, the free fatty acids, not esterified fatty acids, were obtained by the ion-exchange chromatography from the acetone soluble fractions; *viz.* 5.31 g. of the raw material dissolved in a concentration of about 0.3 per cent in absolute ethanol and let this solution pass through a column (1 × 100 cm.) of 60 g. of anhydrous, regenerated Amberlite IRA 410 (30 to 50 mesh) packed with absolute ethanol, at a flow rate of 50~60 ml. per hour. The effluent corresponded to neutral fats, the yielding being 1.05 g. and acid value 0. The column then was eluted with 0.01 *N*solution of potassium hydroxide in 95 per cent ethanol. The elute was concentrated, acidified and extracted with ether. Finally 4.16 g. of a brown semi-solid mass were obtained. The physical and chemical properties of the fatty acids originated from these 5 bacteria are given in Table II.

Chromatographic Analysis of the Bacterial Fatty Acids—Seventy to 100 mg. of each sample of the above fatty acids mixture were at first put through the oxidation and hydrogenation. The resulting chromatograms are seen in Fig. 2 to 6, and the numerical data obtained from these chromatograms are tabulated in Table III to VIII.

From the summarized results tabulated in Table VIII, we see that among the common fatty acids palmitic and unsaturated C_{18} acids are present most abundantly. The latter unsaturated acid is most probably oleic acid. Beside these common acids, quite special acids (see the last column), *viz.* C_{15} acid of *B. Natto* and unsaturated C_{16} acids of the other four specimens were found. The nature of the saturated C_{15} acid is unknown, but its abundance may require further special attention. As for the unsaturated C_{16} acid, the existence of palmitoleic acid may be thought, because this acid is one of the usual acids found in bacterial fatty substance.

TABLE VIII

Summarized Results of the Bacterial Fatty Acids

	Ordinary Fatty Acids		Peculiar Fatty Acids (%)
	Saturated (%)	Unsaturated (%)	
B. alcaligenes faecalis	lauric 3 myristic 3 palmitic 43	unsaturated C_{18} acid 28[1)]	unsaturated C_{16} acid 21[2)]
S. pullorum	C_{10}—C_{12} 17 myristic 12 palmitic 47	unsaturated C_{18} acid 13	unsaturated C_{16} acid 9
B. fluorescens	C_{10}—C_{12} 15 myristic 4 palmitic 32	unsaturated C_{18} acid 31	unsaturated C_{16} acid 19
S. typhimurium	C_{10}—C_{12} 18 myristic 8 palmitic 32	unsaturated C_{18} acid 19	unsaturated C_{16} acid 29
B. Natto	palmitic 11	——	sat'd C_{15} 74 unsat'd C_{15} 14

1), 2): These values are those of column (V) in Table III.

DISCUSSION

Mention must be here made that in the reversed-phase column chromatography a peak does not necessarily mean the presence of a single acid, but sometimes it represents a mixture of two or more fatty acids, *e.g.* even in the case of model experiment, in ss-C_{14} the coexistence of myristic and linoleic acids was experienced. The state indicates that the elution rate of fatty acid is influenced by various factors, not only by the number of carbon atoms but by such factors like nature, number or position of unsaturated bond, or also by the presence of hydroxyl or carbonyl radical. It remains, therefore, to be undecided by this method that myristic acid so listed in the above Tables means really *n*-tetradecanoic acid alone or some other compound; a similar argument can be applied to C_{18} unsaturated acid regarded as oleic (9-octadecenoic) acid and there is a possibility of this being petroselinic (6-octadecenoic) acid, having similar chromatographic behaviour (*3*).

With regard to the (V) column, Table III, one finds such figures of 21 per cent for ss-C_{16} and 28 per cent for ss-C_{18}, which certainly indicate that 21 per cent of unsaturated C_{16} and 28 per cent of unsaturated C_{18} acids are present in the original sample, but one can hardly say how much of them are derived exactly from ss-C_{14} and ss-C_{16} listed in the (IV) column. Yet if the hydrogenation experiments were omitted, *i.e.* without information about the column (III), there would be no way of detecting the carbon numbers of the unsaturated acids (see column (IV)), and consequently this fact makes the hydrogenation indispensable.

The Bertram oxidation gave compatible results in the model experiment. In the more complex natural fatty acids mixture, however, this procedure does not entirely exclude a possibility that only the unsaturated fatty acids were decomposed quantitatively and the saturated fatty acids remained completely unchanged and recovered at a high yield. With regard to the hydrogenation, one may remember the existence of substance such as lactobacillic acid, which is decomposed by using platinum oxide.

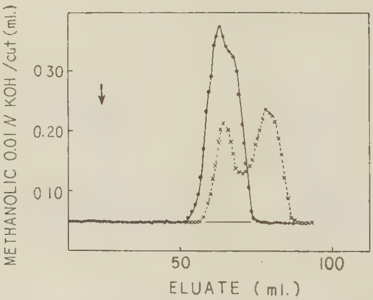

FIG. 7. Showing the effect of acetone content of the developer on a chromatographic behavior of a mixture of capric and lauric acids. Sample was dissolved in 55 per cent aqueous acetone.

—●— developed with 60 per cent aqueous acetone: single asymmetric peak.

—×— developed with 55 per cent aqueous acetone: double peaks.

It would be appropriate to add a few words with regard to the technical points of the chromatography. All the material to be tested were dissolved in 55 per cent aqueous acetone. The ability of the developer depends, in general, greatly on its acetone content within the range of a certain concentration; the higher is the content, the sharper is the peak, but the lesser becomes the resolving power for the components; or conversely, a lower acetone content gives a better resolution, but in such a case the shape of the peak becomes more flatter, therefore, available percentage to be used must be decided after a good many tests, so as to avoid overlooking any peak, moreover, to make enough the shape of the peak sharp—an example is shown in Fig. 7.

In the present investigation, the most suitable concentrations were found to be 60 per cent for ss-C_{12}, 65 per cent for ss-C_{14}, 70 per cent for ss-C_{16} and 75 per cent for ss-C_{18}—of course these depend upon the volume of the column employed.

SUMMARY

1. On trial analysis of a synthetic fatty acid mixture (using the material in three forms, the original non-treated, oxidized and hydrogenated), reversed-phase column chromatography is shown to be employed for the analysis of a fatty acids complex, provided that the material could be available in a quantity of 200 to 300 mg.

2. Fatty acids obtained from *B. alcaligenes faecalis*, *S. pullorum*, *B. fluorescens*, *S. typhi murium* and *B. Natto* were analysed by this method.

3. Palmitic and unsaturated C_{18} acids were found to be the main components. Unsaturated C_{16} acids were also found to some extent. Among the unsaturated C_{16} and C_{18} acids the amount of palmitoleic and oleic acids seemed to be predominant.

4. A saturated C_{15} acid was found abundantly in the fat of *B. Natto*.

The author wishes to thank Prof. S. Akashi, for the gift of the material and his interest during the present work.

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STUDIES ON BACTERIAL FATTY ACIDS

STRUCTURE OF SUBTILOPENTADECANOIC AND SUBTILOHEPTADECANOIC ACIDS*

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(Received for publication, June 22, 1959)

Subtilopentadecanoic and sutiloheptadecanoic acids are the chief fatty acids components of the neutral fat obtained from *B. subtilis* and can be isolated by the reversed-phase column chromatography on preparative scale. In the present paper it is shown that these acids are C_{15} *iso*- and C_{17} *iso*- types, *viz.*, 13-methyltetradecanoic and 15-methylhexadecanoic acids, respectively.

EXPERIMENTAL

Materials—The neutral fat under consideration was prepared from a certain strain of *B. subtilis* and kindly given to the author by Prof. S. Akashi. Six point one g. of the neutral fat were saponified for 5 hours with 10 volumes of *N* methanolic potassium hydroxide. After removal of the unsaponifiable matter, 3.8 g. of the mixed fatty acids were obtained (yield, 63 per cent). This material was a dark brown semi-solid mass with melting point 30 32°, neutralization value 216.4 and iodine value 23.8.

Preliminary Chromatography—Chromatographic assay of the mixed fatty acids, following the method previously reported (*1*), revealed two well-defined peaks (see SS-C_{15}** and SS-C_{17} in Fig. 1 (A) and (B)), which came between those of myristic and palmitic acids, and of palmitic and stearic acids respectively—indicating strongly that these were corresponding to C_{15} and C_{17} acids. The oxidation of the unsaturated acids by Bertram procedure resulted in the third chromatogram reproduced in the same figure.

A detailed comparison of (A) and (C) led to a conclusion that 80 per cent or more of the mixed acids was composed of SS-C_{15} and SS-C_{17}, mainly of saturated type (see Table I), and the names 'subtilopentadecanoic' and 'subtiloheptadecanoic' acids are proposed for these saturated fatty acids.

Reversed-phase Column Chromatography on Preparative Scale and Isolation of Subtilopentadecanoic and Subtiloheptadecanoic Acids—In order to isolate the desired fatty acids by the reversed-phase column chromatography, some model experiments were undertaken by the method originated by Silk and Hahn (*2*). The column employed was of the dimension of 3×100 cm., and 300 g. of liquid paraffin treated non-wetting kieselguhr were suspended in 2000 ml. of 55 per cent aqueous acetone and packed into the column under water pressure

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* A part of this work was done in the Department of Biochemistry, Faculty of Medicine, Nagoya City University, Nagoya, and was read before The 4 th Symposium on Bacterial Toxin held in Hakone, August 9 th, 1957 and The 31 st Meeting of The Japanese Biochemical Society held in Sapporo, July 15 th, 1958.

** The abbreviation SS-C_{15}, or others, is the symbol denoting a peak in chromatogram including all the fatty acids which can be eluted together with saturated straight chain C_{15} acid.

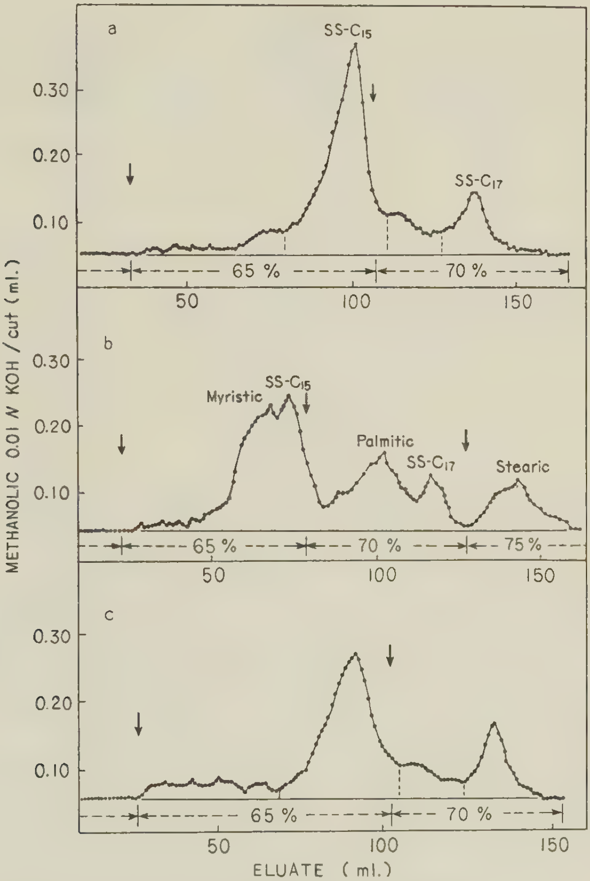

FIG. 1. Chromatographic separation of the fatty acids of *B. subtilis* on reversed-phase column (1×100 cm.).

(a) Fifteen mg. of the fatty acids of *B. subtilis*. (b) Synthetic mixture of the fatty acids of *B. subtilis* with 3.5 mg. of myristic, 4 mg. of palmitic and 4.5 mg. of stearic acids. (c) Thirteen mg. of the fatty acids of *B. subtilis* oxidized by Bertram procedure. Samples were dissolved in 55% aqueous acetone and chromatographed. Eluting solvents were changed at the arrow sign. Recoveries were more than 97%.

of 250 cm. The column was left for about 2 days under normal pressure to ensure complete packing. The same column could be used for 10 successive runs of chromatography. The rate of flow was maintained at approximately 8 ml. per minute. A typical separation is shown in Fig. 2 (A), (B) and (C).

The titration value was obtained with an aliquot of each eluate, as was indicated in the footnote of the figure. The remainder of the sample was used for the isolation of the fatty acids. Such experiments indicated the possibility that those acids coming within the range $C_{12}\sim C_{18}$ can be separated, as have been done with the small scale chromatography, provided that each constituent exists in an amount less than 200 mg. With normal saturated fatty acids, it is perfectly feasible to separate two fatty acids having a difference of two carbon atoms, and in those instances where the difference is one carbon, the separation, though imperfect, is quite possible. With regard to the unsaturated acids, *e. g.* oleic and linoleic acids, the separation can easily be obtained.

TABLE I

Chromatographic Result of the Fatty Acids Prepared from B. subtilis

The Name of Peak	Non-treated Sample (I)%	Oxidized Sample (II)%	Unsaturated Fatty Acids (I-II)%	Fatty Acids Found %	
Lower Peak	9	11		lower	9
SS-C_{15}	64	53 (subtilo-C_{15})	11	subtilo-C_{15} unsat'd SS-C_{15}	53 11
SS-C_{16}	10	10 (palmitic)	0	palmitic	10
SS-C_{17}	17	15 (subtilo-C_{17})	2	subtilo-C_{17}	15
	100	89[1]			

1) A hundred mg. of the fatty acids were oxidized by the Bertram procedure, with a recovery of 89 mg. (89%).

This procedure was applied to the mixture of fatty acids obtained from *B. subtilis* for the isolation of the two fatty acids under the present consideration. As the first step, the contaminating unsaturated acids and pigmented matter were removed by the Bertram oxidation. One g. of the mixed fatty acids were dissolved by warming in 10 ml. of 1/2 *N* potassium hydroxide and 50 ml. of water, and after cooling the solution, 1 ml. of 50 per cent (weight/volume) aqueous solution of potassium hydroxide and 300 ml. of 5 per cent potassium permanganate solution were added with continuous shaking. The mixture was allowed to oxidize for 18 hours at 18°. It was then acidified with 2 ml. of 50 per cent sulfuric acid, decolorized with saturated sodium hydrosulfite solution, concentrated under reduced pressure and extracted with petroleum ether (30–60°). The petroleum ether layer was washed with 5 per cent sodium bicarbonate solution and water, and then dried. After removal of petroleum ether, the final oxidation product was obtained as a white mass, the average yield being 83 per cent.

The next stage was the chromatography, on a preparative scale, of the oxidation products. About 400 mg. of this oxidized material, dissolved in 300 ml. of warm 55 per cent aqueous acetone, was applied to the column (see above) and the elution was accomplished with 70 and 75 per cent aqueous acetone. A typical chromatographic separation is shown in Fig. 3.

The chromatographic process was repeated 7 times in succession, and the portions indicated by the horizontal bars in Fig. 3 were collected. Each fraction was concentrated, acidified and extracted with ether. The ether layer was washed and dried. After removal of ether, a white waxy substance was obtained. The yield was 1.1 g. for subtilopentadecanoic acid, and 0.92 g. for subtiloheptadecanoic acid. These fractions, however,

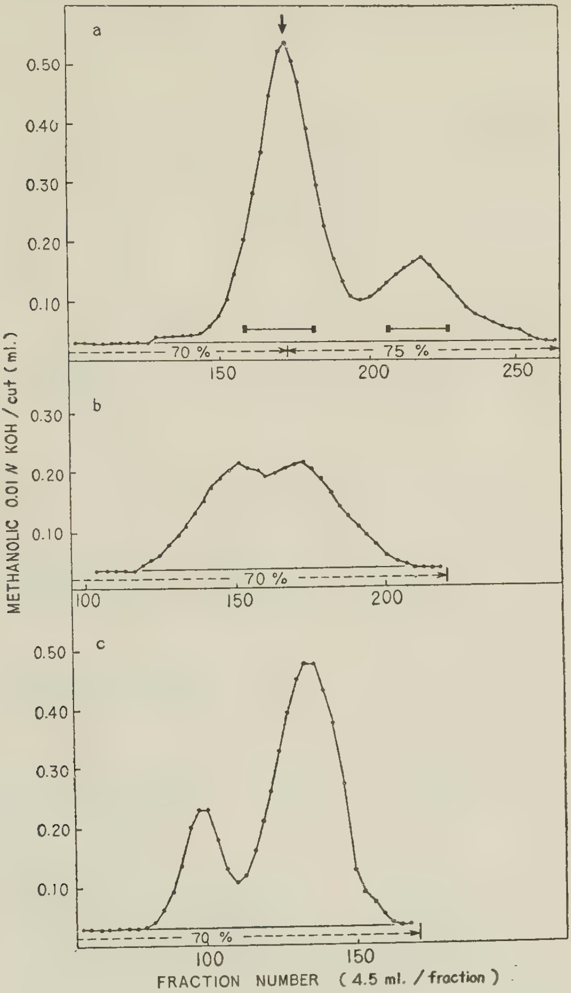

Fig. 2. Chromatographic separation of the fatty acids on preparative column (3 × 100 cm.).

(a) Synthetic mixture of 200 mg. of myristic and 80 mg. of palmitic acids. (b) Synthetic mixture of 100 mg. of myristic and 100 mg. of *n*-pentadecanoic acids. (c) Synthetic mixture of 80 mg. of oleic and 200 mg. of linoleic acids. Samples were dissolved in 55% aqueous acetone and chromatographed. The volume of each tube was 4.5 ml. and the titrations were performed on 0.5 ml. aliquot from every third collections.

were still contaminated with liquid paraffin used as the stationary phase, and so the fatty acids were converted into potassium soaps, and liquid paraffin was removed by extraction with ether. The fatty acids reconverted from soaps with 6*N* hydrochloric acid were repeatedly recrystallized from ethanol and acetone until they showed the fixed melting points. Thus, 600 mg. of subtilopentadecanoic acid and 175 mg. of subtiloheptadecanoic acid were obtained. The physical and chemical constants of the two fatty acids thus prepared are shown in Table III.

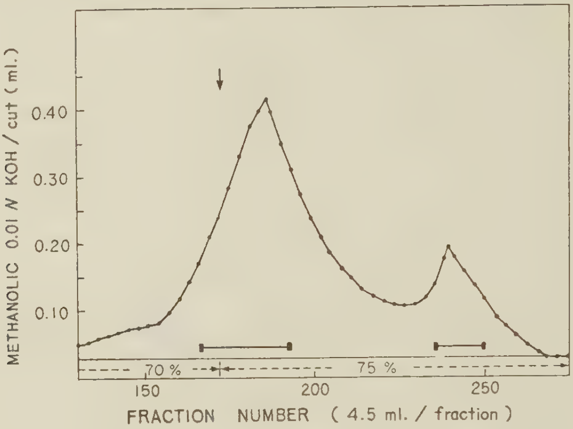

FIG. 3. Chromatographic separation of the fatty acids of *B. subtilis* oxidized by Bertram procedure.

About 400 mg. of the fatty acids dissolved in 55% aqueous acetone were applied and eluted with 70 and 75% aquous acetone. The corresponding portions to the marked lines were collected to isolate subtilopentadecanoic and subtiloheptadecanoic acids.

Infra-red Spectrum—The spectra were obtained by courtesy of Dr. Koji Nakanishi, Faculty of Science, The Tokyo Kyoiku University. Hilgar's H 800 (sodium chloride prism) was employed, using the potassium bromide pellet method. A portion of it is reported in Fig. 4.

X-ray Diffraction—The characteristic long spacings for the fatty acids were kindly measured by Dr. Yohichi Iidaka, Mineralogical Institute, Science Department, University of Tokyo, with a Norelco X-ray Diffractometer, no filtered chromium K_α radiation being used. The samples were pressed on the glass slides.

Specific Rotation—A Rudolph polarimeter was used.

Subtilopentadecanoic acid—$\alpha=+0.037$ d=1, c=0.861
(8.61 mg./ml. of $CHCl_3$)
$\alpha=+0.040$ d=2, c=0.900
(9.00 mg./ml. of $CHCl_3$)
Subtiloheptadecanoic acid—$\alpha=+0.041$ d=1, c=0.853
(8.53 mg./ml. of $CHCl_3$)
$\alpha=+0.040$ d=2, c=0.888
(8.88 mg./ml. of $CHCl_3$)
Palmitic acid (Kahlbaum)—$\alpha=+0.039$ d=2, c=0.814
(8.14 mg./ml. of $CHCl_3$)

From these findings, it is clear that the specific rotations are nearly zero within the experimental error.

Syntheses of Normal Pentadecanoic and Heptadecanoic Acids—They were synthesized from commercial cetyl alcohol by the nitrile synthesis (*3*). Further purification was effected by the column chromatography on a preparative scale. The purity of these synthesized acids can be judged by the constants presented in Table II, when these are compared with those quoted in Table III.

TABLE II

Physical and Chemical Constants of the Synthesized n-Pentadecanoic and n-Heptadecanoic Acids

Acid	M. P., °C.	N.V.	I. V. (Yasuda)	$[\alpha]_D$	Long Crystal Spacings, Å	Progression Bands
n-C_{15}	52.5-53.0	232.1	0	0	35.92	8
n-C_{17}	61.0-61.5	208.8	0	0	40.26	9

The results are strongly indicative of the extreme purity of the synthesized normal acids.

TABLE III

Comparison of Subtilopentadecanoic and Subtiloheptadecanic Acids with n- and iso-Pentadecanoic and Heptadecanoic Acids

Acid	Elementary Analysis, %	N. V.	I. V. (Yasuda)	$[\alpha]_D$	M. P., °C.	Long Crystal Spacings, Å	IR (Doublet near 1380 cm^{-1})
n-C_{15}	$C_{15}H_{30}O_2$. (242) Cald. C: 74.32 H: 12.48	231.6	0	0	[1] 52.3–53.3	[4] 35.75	—
iso-C_{15}					[2] 51.0–51.5	[5] 29.8	+
Subtilo-C_{15}	Found* C: 74.61 H: 12.55	233.0	0	0	51.8–52.3	29.82	+
n-C_{17}	$C_{17}H_{34}O_2$. (270) Cald. C: 75.50 H: 12.67	207.6	0	0	[3] 61.3–	[3] 40.45	—
iso-C_{17}					[2] 59.5–60.0	[5] 33.35	+
Subtilo-C_{17}	Found* C: 75.61 H: 12.75	208.8	0	0	60.5–61.0	33.31	+

The analytical values on *n*- & *iso*- C_{15} & C_{17} acids are calculated or cited from the following authors;

1) Links, J. & Groot, M. S. de, *Rec. Trav. Chim. Pays-Bas.* **72**, 57 (1953)
2) Weitzel, G. & Wojan, J., *Hoppe-Seyler's Z. physiol. Chem.*, **287**, 65 (1951)
3) Francis, F. & Piper, S. H., *J. Amer. Chem. Soc.*, **61**, 577 (1939)
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* Carbon and hydrogen analyses were performed by the Central Micro Analytical Laboratory, Kyoto University.

RESULTS

The physical and chemical constants of subtilopentadecanoic and subtiloheptadecanoic acids are summarized in Table III and, for comparison, the values of *n*- and *iso*- pentadecanoic and heptadecanoic acids reported by several authors are cited in the same Table.

The IR spectra (Fig. 4) showed that the present two acids revealed a doublet (wave numbers: 1367 and 1385 cm^{-1}) and an absorption at 1175 cm^{-1}, which are attributed to the presence of isopropyl radical—normal acids give no such absorptions. It should be noted that the appearance of the doublet around 1380 cm^{-1} is not the sole property attached to *iso*-acids but to *neo*-acids too; the melting points of the *neo*-acids are lower than those of *iso*-acids, so the possibility of the presence of the *neo*-acids in the present fatty acids is excluded. Between the wave numbers 1180–1350 cm^{-1} there were oscillatory

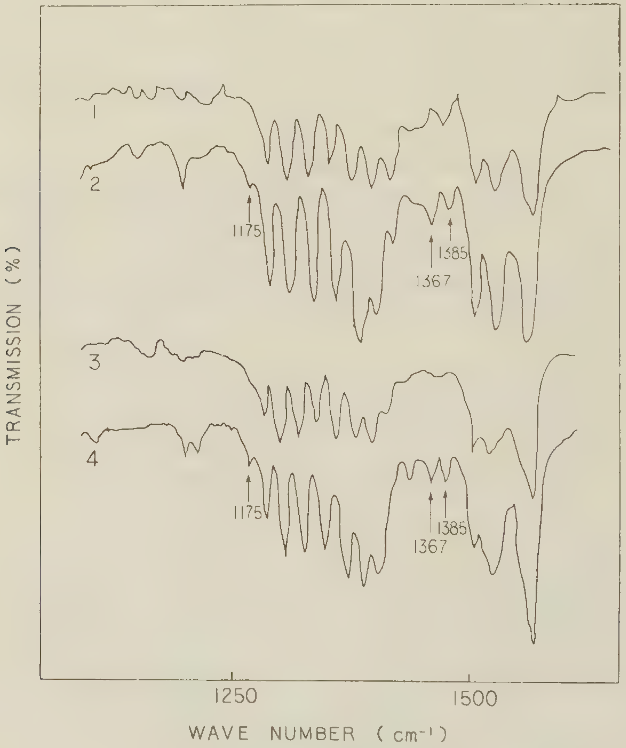

Fig. 4. Infrared spectrum of the fatty acids.

Spectrum 1. *n*-pentadecanoic acid, Spectrum 2. subtilopentadecanoic acid, Spectrum 3. *n*-heptadecanoic acid and Spectrum 4. subtiloheptadecanoic acid. Hilgar's H 800 (sodium chloride prism) was employed, using the potassium bromide pellet method (1 mg. of the material per 200 mg. of the potassium bromide).

variations in absorption, the progression bands, and subtilopentadecanoic acid showed 7 absorptions and subtiloheptadecanoic acid 8, while corresponding normal acids show, according to Meiklejohn *et al.* (*4*), 8 and 9 respectively. Beside these fiindings no other peculiality was noticed except the absence of any cyclic ring compounds.

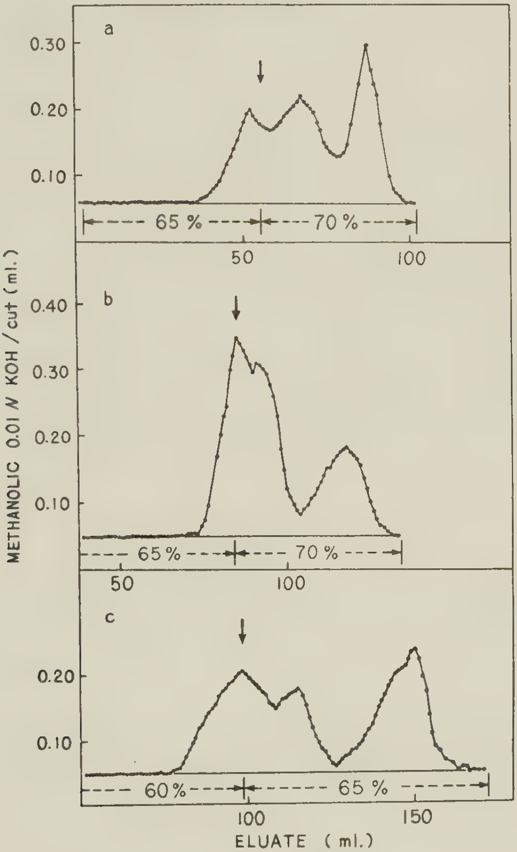

Fig. 5. Chromatographic separation of the fatty acids on reversed-phase column (1×100 cm.).

(a) Synthetic mixture of 4 mg. of myristic, 4.5 mg. of *n*-pentadecanoic and 5 mg. of palmitic acids. (b) & (c) Synthetic mixture of 4.4 mg. of myristic, 4.6 mg. of subtilopentadecanoic acid and 5 mg. of palmitic acids. Samples were dissolved in 55% aqueous acetone and chromatographed. Elution was done with 65 and 70% aqueous acetone in (a) and (b) and with 60 and 65% in (c).

With regard to the long spacings, it is seen that with subtilopentadecanoic acid the value is quite different from that of normal C_{15}, and is the same with that of *iso* form, and that the same statement is true in the case of subtiloheptadecanoic acid. In conclusion, it is clear that subtilopentadecanoic and subtiloheptadecanoic acids are C_{15} *iso*- and C_{17} *iso*- acids—13-methyltetradecanoic and 15-methylhexadecanoic acids respectively.

The key point, however, in the determination of the structure of the acids would be that they are normal acids or *iso*-forms, for melting points of *iso*-acids are less than 1° below those of the corresponding normal acids. Accordingly, the present two fatty acids were compared with the synthesized normal C_{15} and C_{17} acids. When subtilopentadecanoic acid was mixed with an equal quantity of synthesized *n*-C_{15} acid the melting point of the latter (52.5–53.0°) was depressed by 5.5°, and subtiloheptadecanic acid, on admixture with synthesized *n*-C_{17} acid, m. p. 61.0∼61.5°, showed depression of melting point by 5.0°. These depression are in accord with depression of 5–7° found when odd-numbered normal acid and *iso*-acids are mixed. The comparison of chromatography of *n*-C_{15} acid and subtilopentadecanoic acid (see Fig. 5), indicates a certain difference in their behavious; thus the peak of *n*-C_{15} acid appeared roughly in the center between those of myristic and palmitic acids, whereas subtilopentadecanoic acid deviated more to the side of the peak of myristic acid—though the mixture of these two C_{15} fatty acids showed a single peak and no sign of separation was observed. These findings can be expected in the other pair, *i. e.* *n*-C_{17} and subtiloheptadecanoic acids. In essence, one may regard the two fatty acids in question not to be classified as normal acids.

DISCUSSION

It is generally assumed that the occurrence of odd-numbered or branched fatty acids is a sort of rarity in nature, and within the scope of literature available to the present author, there have been reported the following instances.

1) *In micro-organisms*—One can find a few instances, *e. g.* tuberculostearic or lactobacillic acids (*5*). To these, there are recent reports by Akashi and the present author of the presence of C_{15} acids in the fat of *Sarcina** and *B. Natto* (*1*).

2) *In degras*—Weitkamp (*6*) reported 12 odd-numbered *anteiso*-acids, together with 10 even-numbered *iso*-acids.

3) *In preen gland*—Methylhexanoic acids were isolated from the preen glands of the duck and ostrich by Weitzel *et al.* (*7*).

4) *In mammalian fat*—Shorland and others (*8*) isolated the several fatty acids of normal, *iso*- and *anteiso*-series but their contents were of the order of 1 per cent.

5) *In plant*—Sterculic acid (*9*) has been found in *Sterculia faetida.*

The present odd-numbered *iso*-acids seem to be more rare occurrence, and have mere three allied members, *viz.* C_{13} and C_{15} acids of Shorland *et al.* (*10*, *11*) found in butter fat and C_{17} acid in ox fat reported by Hansen

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* in press

et al. (*12*). This rarity of occurrence and a peculiarity of percentage composition in the total fat—the three cited acids constitute only minute amount of the order of 0.1 per cent, while the present two acids form a bulk of the fatty acids of *B. subtilis*—may lead one to venture into a speculation that the odd-numbered *iso*-acids in the animal fat owe their origins from such sources as the present bacterial fat. Whatever these may be, it would be interesting to ponder on the probable biological or rather evolutional meaning of these rare fatty acids.

SUMMARY

1. Subtilopentadecanic and subtiloheptadecanoic acids constitute a great proportion of the fatty acids obtained from *B. subtilis*, being roughly 50 and 15 per cent respectively.

2. The two acids were isolated by reversed-phase column chromatography and found to be C_{15} *iso*- and C_{17} *iso*-acids, *v.z.* 13-methyltetradecanoic and 15-methylhexadecanoic acids.

The author wishes to thank Prof. S. Akashi, for the gift of the material and his interest in this work and to Dr. K. Nakanishi and Dr. Y. Iidaka for the measurement of infrared and X-ray diffraction, and to Prof. D. J. Hanahan, for his kindness in reading this manuscript.

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BIOCHEMICAL STUDIES ON THE FORMATION OF THE SILKPROTEIN

VII. THE CONVERSION OF GLYCINE TO SERINE IN THE SILKWORM LARVA

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In mammals, Winnick *et al.* (*1*) incubated the homogenate of rat liver with glycine-2-C^{14} and found that the radioactive serine was synthesized. Siekevitz and Greenberg (*2*), using the liver slice, found that glycine-1-C^{14} was converted to serine-1-C^{14} and glycine-2-C^{14} to serine-2–3-C^{14}. Sakami (*3*) gave proof of the conversion of glycine-2-C^{14} to serine-2–3-C^{14} *in vivo*. Sakami (*4*) reared rats on food containing glycine-1-C^{13} and C^{14}-formic acid, and found that serine isolated from the liver had C^{13} in the position of the carbon of carboxyl group, and C^{14} in the position of carbon-2. He demonstrated that formic acid is an intermediate in this reaction. At present, the mechanism of this conversion in mammals is explained as follows: Serine is formed by condensation between the formaldhyde derived from glycine *via* glyoxylic acid and another molecule of glycine. The conversion of glycine, however, to glyoxylic acid has not been proved experimentally, because glyoxylic acid does not usually exist in rats.

It has been pointed out that glyoxylic acid exists in a comparatively high content in the body fluid (*5*, *6*) of silkworms, and that serine must also be in a comparatively high amount synthesized in the silkworm body (7). Silkworm, therefore, seems to be one of the most suitable organism for the study of the conversion of glycine to serine.

Serine is contained somewhat abundantly in cocoon fibres produced by silkworms, about 15 per cent in the fibroin, and about 30 per cent in the sericin. A clue, therefore, to the biosynthesis of serine seems to be of special importance in relation to the formation of silk. Little is known, however, of the precursors of serine synthesis in the silkworm larva.

The current work was performed, using glycine-1-C^{14} and glycine-2-C^{14}, to clarify whether glycine is one of the most important precursors of serine synthesis in the silkworm.

EXPERIMENT

1. Injection of Glycine-1-C^{14} and Glycine-2-C^{14} to Silkworms—Glycine-1-C^{14} (1.44 mc. per mmole) and glycine-2-C^{14} (3.87 mc. per mmole) were obtained from the Radiochemical Centre, Amersham, England. A half μc. of radioactive glycine per worm was injected into the silkworms, Nichi 122×Si 115, at the fourth day of the fifth instar. These silkworms were reared on mulberry leaves until they produced cocoons.

2. *Isolation of Serine, Alanine, Tyrosine and Glycine in Fibroin and Sericin*—The isolation of fibroin and sericin from cocoon fibres was carried out by the procedure described in a previous paper (*8*). Each protein was hydrolyzed with 3 parts of 12*N* hydrochloric acid, and from the hydrolysate tyrosine, glycine, alanine, and serine were isolated in this order by procedures described by Stein and Moore (*9*). Each amino acid obtained was recrystallized until its specific activity became constant. The chemical purity of these amino acids was proved by paper chromatography.

3. *Isolation by Paper Chromatography of Serine in the Fibroin*—Five hundred mg. of radioactive fibroin was hydrolyzed with 20 ml. of 10 per cent hydrochloric acid for 8 hours. Tyrosine was removed directly from the hydrolysate, and then glycine removed as a salt of 5-nitronaphthalene-1-sulfonate. The sulfonate existing in the solution was completely removed by adding 10 per cent sulfuric acid, and the filtrate was concentrated. The residue was analyzed by paper chromatography using phenol-ammonia. After the phenol on the chromatogram was removed by ether, the serine located on the chromatogram was eluted by water (40°). This procedure was repeated until pure serine was obtained.

4. *Degradation of Amino Acids*—Serine and glycine obtained from the silkproteins were degraded according to the procedure described by Vernon *et al.* (*10*).

5. *Isolation of Glyoxylic Acid from the Body Fluid of Silkworms*—One tenth μc. of glycine-1-C^{14} per worm was gived to the silkworms at the fourth day of the fifth instar, Si 115× Nichi 122. After these silkworms were reared on mulberry leaves for 24 hours, about 10 ml. of body fluid was collected from the silkworms and were added to 100 ml. of 10 per cent trichloracetic acid. After standing two hours, the mixture was filtered. To the filtrate were added 50 mg. of potassium glyoxylate as a carrier and 30 ml. of the solution of 2,4-dinitrophenylhydrazine in 2*N* hydrochloric acid. After standing a few hours, the hydrazone of glyoxylic acid was isolated by the procedure described in the previous paper (*5, 6*). Its identification was carried out by paper chromatography, and by analysis of the absorption spectra, in comparison with the authentic compound of glyoxylic acid hydrazone.

RESULTS

Specific activities of serine, alanine, tyrosine, and glycine isolated from the fibroin and the sericin are shown in Table I. The radioactivity was measured with SC-16 windowless gas flow counter (Tracerlab. Inc.). The radioactivity was found in serine, alanine, and glycine, but not in tyrosine. Then, to confirm still more the existence of radioactivity in the amino acids, the glycine, the alanine and the serine were analyzed by paper chromatography. Fig. 1 shows that comparatively high radioactivity is recognized at the position of each amino acid. Fig. 1 was obtained by scanning the chromatogram for radioactivity by the SC-16 windowless gas flow counter.

The amino acids isolated from the fibroin obtained when glycine-1-C^{14} and glycine-2-C^{14} were employed were degraded according to the procedure described by Vernon *et al.* (*10*) in order to know the distribution of C^{14} in the molecule of each amino acid. As shown in Table II, comparatively higher concentration of C^{14} appeared, when glycine-1-C^{14} was employed, in the carbon of the carboxyl group of serine, and a low concentration in the position of the carbon-2 and -3 atom. On the contrary, when glycine-2-C^{14} was employed, the C^{14} appeared chiefly in the position of the carbon-2 and -3 atom of serine, and a low concentration in the carbon of the carboxyl group.

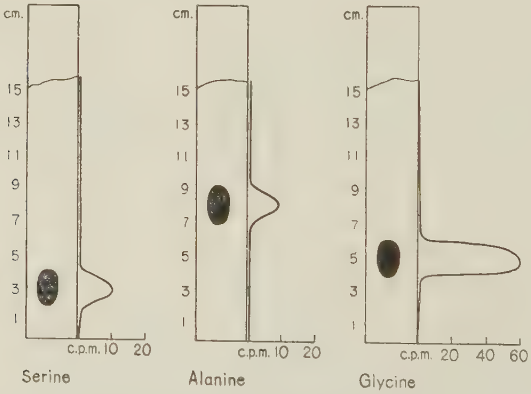
TABLE I

Radioactivity of the Amino Acids isolated from Fibroin and Sericin Produced by Silkworms Which Consumed Glycine-1-C^{14} and Glycine-2-C^{14} at the Fourth Day of the Fifth Instar.

c.p.m./m mole

Amino Acid	Glycine-1-C^{14}		Glycine-2-C^{14}
	Fibroin	Sericin	Fibroin
Serine	4,305[1)]	557[2)]	6,615[3)]
Alanine	1,420	–	1,531
Tyrosine	0	0	0
Glycine	72,825	19,875	187,500

1) The value when 50 mg. of DL-serine was added as a carrier
2) ,, 90 mg. ,,
3) ,, 110 mg. ,,



Serine Alanine Glycine

FIG. 1 Distribution of radioisotope on the chromatogram of the amino acids isolated from the fibroin produced by the silkworms which consumed glycine-1-C^{14} at the fourth day of the fifth instar.

Fig. 2 shows that serine isolated by paper chromatography from the acid hydrolysate of the fibroin when glycine-1-C^{14} or glycine-2-C^{14} was employed has radioactivity in its molecule. Fig. 2 was obtained by scanning the radioactivity on the chromatogram of the serine. Also a radioautograph obtained by superposition of an X-ray film for a few weeks on the chromatogram of isolated serine gave an image at the position of the serine (Fig. 3).

From the experimental results stated above, it may be concluded that

TABLE II

Distribution of the Radioactivity in the Amino Acid Molecule

Amino Acids isolated	C^{14} Amino Acids given to the Silkworm	
	Glycine-1-C^{14}	Glycine-2-C^{14}
	c.p.m./mg.	c.p.m./mg.
Serine		
C-1 (-COOH)	8	2
C-2 (-CHNH$_2$)	2	4
C-3 (-CH$_2$OH)	1	4
Glycine		
C-1 (-COOH)	314	3
C-2 (-CH$_2$NH$_2$)	0	556

Radioactivities of the C-1 and the C-2 of the serine, and the C-1 of the glycine were determined as barium carbonate; and the radioactivities of the C-3 of the serine, and the C-2 of the glycine as formdimedon.

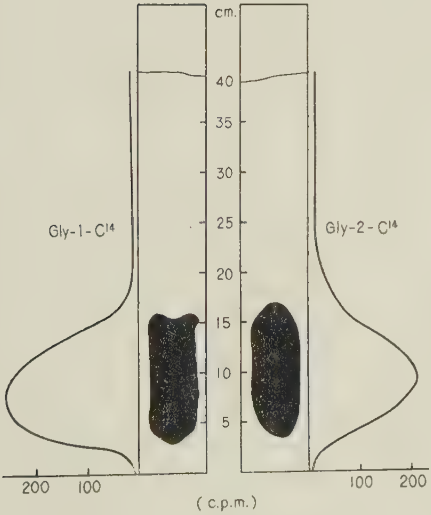


FIG. 2. Distribution of radioactivity on the chromatogram of the serine isolated by paper chromatography from the acid Hydrolysate of the fibroin produced by the silkworms which consumed C^{14} glycine at the fourth day of the fifth instar (Solvent of paper chromatography: phenol containing ammonia).

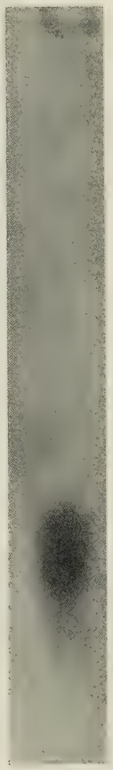


FIG. 3. Radioautograph of the serine isolated from the acid hydrolysate of the fibroin in the case that glycine-1-C^{14} was employed.

the serine in silkworms is partly synthesized from glycine, the carbon of the carboxyl group of glycine molecule is chiefly used for formation of the carbon of the carboxyl group of serine, and the carbon-2 of glycine for formation of the carbon-2 and -3 of serine.

Next, an experiment was carried out to clarify a mechanism of the conversion of glycine to serine. It was investigated whether C^{14} of the glycine-1-C^{14} is used for glyoxilic acid synthesis in the silkworm. After glycine-1-C^{14} was given to the silkworms, glyoxylic acid was isolated from its body fluids as a salt of 2,4-dinitrophenylhydrazine. This result is shown in Table III.

TABLE III

Conversion of Glycine to Glyoxylic Acid

	Hydrazone of Glyoxylic Acid
Specific Activity	5,334 c.p.m./mmole
R_f Value[1]	0.23
Maximum Absorption peak[2]	448 mμ
Yield	20 mg.

1) *n*-Butanol saturated with water was used as a solvent for the analysis of paper chromatography.

2) The measuring of the maximum absorption peak was carried out with Beckman Spectrophotometer in 1.25*N* sodium hydroxyside solution in which the hydrazone of glyoxylic acid is dissolved.

A comparatively high radioactivity was recognized in the hydrazone of glyoxylic acid. This fact seems to suggest that glyoxylic acid is an intermediate in the conversion of glycine to serine in the silkworms.

In the previous paper (*6*), the existence of glyoxylic acid in the silkworm, and the conversion of glyoxylic acid to glycine in silkworms have already been clarified. Recently in our laboratory, it was found that the carbon of formate is utilized for serine synthesis in the silkworm (unpublished) and that serine is converted to glycine in silkworms (*11*). From these experimental results, the mechanism of the conversion of glycine to serine seems to be as follows. The mechanism of this conversion obtained in silkworms agrees favourably with that of rats.

$$\underset{\text{Glycine}}{CH_2NH_2\cdot COOH} \rightleftarrows \underset{\text{Glyoxylic Acid}}{CHO\cdot COOH} \;;\quad CHO\cdot COOH \rightarrow HCHO + CO_2$$

$$\underset{\text{Formaldehyde}}{HCHO} + \underset{\text{Glycine}}{CH_2NH_2\cdot COOH} \rightleftarrows \underset{\text{Serine}}{CH_2OH\cdot CHNH_2\cdot COOH}$$

In this paper, it is shown that the serine in the silkproteins, the fibroin and the sericin, is not only derived from the mulberry leaves which were digested by the silkworms, but is also derived from the serine that was synthesized from the glycine in the silkworm body. However glycine must be also synthesized in the silkworm body (*5*, *6*). A clue, therefore, to the mechanism of the serine synthesis must be studied in future in close connection with the glycine synthesis in silkworms.

It is shown also in the present study that glycine is used for alanine synthesis in silkworms.

SUMMARY

1. To clarify the conversion of glycine to serine in the silkworm, glycine-1-C^{14} and glycine-2-C^{14} was given *per os* to the silkworms, and then serine was isolated from the fibroin and the sericin produced by these silkworms. The serine had a comparatively high radioactivity in its molecule.

2. The carbon of the carboxyl group and the carbon-2 atom of glycine are respectively used for synthesis of the carbon of the carboxylic group and the carbon-2 and -3 atom of serine in the silkworm.

3. It was found that glycine is converted to glyoxylic acid in the silkworm by clarifing the existence of radioactivity in the glyoxylic acid which was isolated from the body fluid of the silkworms to which glycine-1-C^{14} was given *per os*.

4. These facts seem to suggest that serine which is contained in comparatively high amount in fibroin (15 per cent) and in sericin (30 per cent) is derived not only directly from the serine in the mulberry leaves eaten by silkworm, but also from the serine which was synthesized from the glycine in the silkworm body.

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THE DIFFERENCES IN SOME LABILE CONSTITUENTS AND SOME ENZYMATIC ACTIVITIES BETWEEN THE RED AND THE WHITE MUSCLE*

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Since the classic study of Ranvier (*1*), the striated muscles of many animals have been generally classified into two types, *i.e.*, the red and the whith muscle. However, the histological study of Denny-Brown (*2*) showed that the striated muscle of mammals was composed of an intimate mixture of the red and the white muscle fiber, and the red or the white muscle was merely composed of an excessive amount of red or white muscle fibers.

The biochemical differences between the red and the white muscle have been reported by some investigators. Riesser (*3*) showed that the white muscle of rabbits contained more creatine and creatinine compared with the red. Embden *et al.* (*4*) reported that the red muscle contained more cholesterol than did the white. Haurowitz *et al.* (*5*) ascribed the red colour of muscle to myoglobin and cytochromes contained in the muscle fibers. Lawrie (*6*) demonstrated a close correlation in the amounts of succinic dehydrogenase, cytochrome oxidase and myoglobin present in three skeletal muscles of the horse. In all those studies, however, the muscle preparations used for quantitative estimation were not pure materials, and contained some amounts of the other types of muscle fibers.

In the previous communications (*7–9*), the author revealed from the histochemical study that the striated muscle of mammals contained three types of muscle fibers, *i.e.*, the red, the white, and the medium muscle fibers, which were different in the activities of succinic dehydrogenase, cytochrome oxidase, diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase. It was further observed that the ratios of three types of muscle fibers were different for each muscle and that even in one muscle the distribution of three types of muscle fibers differed markedly with the portions of the muscle. When the quantitative study is made without taking this histological fact into consideration, constant results will not be obtainable. In the present study, the practically pure preparations of red muscle fibers and those of white muscle fibers of rabbits were successfully obtained and the quantitative differences were observed between them in some important labile constituents and some enzymatic activities.

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* The following abbreviations are used in this paper: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; DPN, diphosphopyridine nucleotide; HDP, fructose-1,6-diphosphate; P, phosphate.

EXPERIMENTALS

Tissue Preparation—Healthy adult rabbits were used. The following operation was performed in a cold room at 0°–1°. The animal was killed by decapitation. In the case of the estimation of glycogen and acid-soluble phosphate compounds, the animal was anaesthetized by intraperitoneal injection of 10 ml. of 20 per cent magnesium sulfate solution and 5 ml. of 2.5 per cent sodium pentobarbital solution to prevent the muscular contraction. The muscle was removed as quickly as possible. The central part of *M. soleus* was used as the red fibers, and the posterior edge of *M. adductor magnus* having a width of 1 mm and a length of about 5 cm was used as the white fibers (*vide infra*).

Histochemical Demonstration of Succinic Dehydrogenase—The histochemical demonstration of succinic dehydrogenase was made by the method of Seligman and Rutenberg (*10*).

Analytical Procedures

Glycogen—Glycogen was extracted with 30 per cent KOH solution according to the method of Somogyi *et al.* (*11*). The quantitative determination was made by the anthrone method (*12*).

Acid-Soluble Phosphate Compounds—The tissue sample was quickly thrown into 99 per cent cold ethanol at −15°. Then the aliquot was transferred into a glass homogenizer containing cold 6 per cent perchloric acid solution. The perchloric acid extraction was carried out twice, and then the combined perchloric acid extract was used for the estimation of acid-soluble phosphate compounds. For the determination of inorganic phosphate and creatine phosphate, the method of Ernster *et al.* (*13*) was employed. The total acid-soluble phosphate was determined by the modified method of Fiske-Subbarow as described by LePage (*14*). The *Δ7* phosphate was determined as inorganic phosphate after the hydrolysis of the sample in 1*N* H_2SO_4 at 100° for 10 minutes.

The Assay of Pyruvate Oxidation—The assay system of Potter (*15*) was employed. The reaction mixture contained 0.3 ml. of 0.1 *M* KH_2PO_4 (adjusted to pH 7.3 with KOH), 0.06 ml. of 0.1 *M* K-fumarate, 0.06 ml. of 0.1 *M* K-pyruvate, 0.4 ml. of 0.03 *M* $MgCl_2$, 0.3 ml. of 0.01 *M* K-ATP, 0.51 ml. of H_2O, 0.97 ml. of 0.50 *M* sucrose and 0.4 ml. of 10 per cent muscle homogenate in 0.25 *M* sucrose. The reaction mixture was incubated in Warburg manometer at 37° for 30 minutes under air as gas phase, and the oxygen uptake was estimated.

The Assay of Succinic Dehydrogenase—The assay system of slightly modified method of Potter (*16*) was employed. In the presence of fumarate the oxidation of pyruvate may be adequate for the measurement of activity of tricarboxylic acid cycle. The reaction mixture contained 1.0 ml. of 0.1 *M* phosphate (pH 7.4), 0.3 ml. of 0.5 *M* Na-succinate pH 7.4, 0.4 ml. of 1×10^{-4} *M* cytochrome c, 0.3 ml. of 4×10^{-3} *M* $CaCl_2$, 0.3 ml. of 4×10^{-3} *M* $AlCl_3$, 0.3 ml. of H_2O and 0.4 ml. of 10 per cent muscle homogenate in 0.25 *M* sucrose. The reaction mixture was incubated in Warburg manometer at 37° for 30 minutes under air as gas phase, and the oxygen uptake was estimated.

The Assay of Anaerobic Glycolysis—The method of LePage *et al.* (*17*) was used. The reaction mixture contained 0.30 ml. of 0.024 *M* potassium phosphate (pH 7.6), 0.15 ml.

of 0.5 *M* $KHCO_3$, 0.30 ml. of 0.4 *M* nicotinamide, 0.10 ml. of 0.01 *M* Na-ATP, 0.20 ml. of 0.003 *M* K-DPN, 0.15 ml. of 0.04 *M* K-HDP, 0.30 ml. of 0.10 *M* glucose, 0.20 ml. of 0.10 *M* $MgCl_2$, 0.10 ml. of 0.15 *M* K-pyruvate, 0.15 ml. of 0.20 *M* KF, 0.75 ml. of H_2O and 0.30 ml. of 10 per cent muscle homogenate in 0.154 *M* KCl. The reaction mixture was incubated in Warburg manometer at 37° for 30 minutes under the gas phase of 95 per cent N_2 and 5 per cent CO_2. The reaction was stopped by adding 0.25 ml. of 65 per cent trichloroacetic acid and then the lactic acid in the flask content was estimated by the modified method of Barker and Summerson (*18*).

The Assay of Adenosine Triphosphatase—The method of Potter (*19*) was employed. The reaction mixture contained 0.3 ml. of 0.02 *M* K-ATP pH 7.6, 0.3 ml. of 0.02 *M* $MgCl_2$, 0.3 ml. of 0.02 *M* $CaCl_2$, 0.9 ml. of 0.25 *M* sucrose, 0.2 ml. of 5 per cent muscle homogenate in 0.125 *M* sucrose. After the incubation of the reaction mixture at 37° for 5 minutes, the reaction was stopped by adding 1.0 ml. of 1.5 *M* trichloroacetic acid and liberated inorganic phosphate was estimated by the modified method of Fiske and Subbarow described by LePage (*14*).

RESULTS

The histochemical study of succinic dehydrogenase revealed that the central part of *M. soleus* was completely free of white muscle fiber (Fig. 1) and the posterior edge of *M. adductor magnus* almost free of red muscle fibers (3 per cent or less in volume) (Fig. 2).

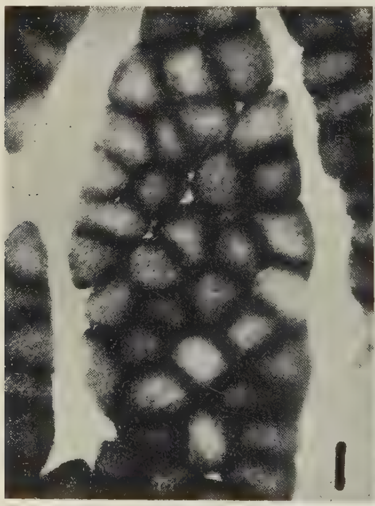

FIG. 1. Cross section of central part of *M. soleus*, succinic dehydrogenase reaction. ×100

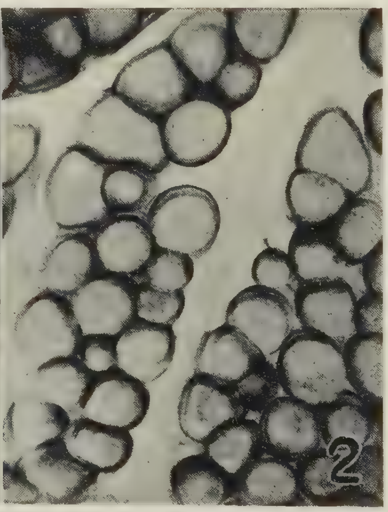

FIG. 2. Cross section of posterior edge of *M. adductor magnus*, succinic dehydrogenase reaction. ×100

Concentration of Glycogen and Acid-Soluble Phosphate Compounds—The difference in content of glycogen and acid-soluble phosphate compounds between the red and the white muscle was shown in Table I. The marked difference in glycogen content was observed between the two types of muscles. The results of 16 serial experiments showed that the white muscle contained about 3.7 times as much glycogen as the red.

TABLE I

Concentration of Glycogen and Acid-Soluble Phosphate in the Red and the White Muscle of Rabbits

Constituent	Expt. No.	Red muscle M±σ	White muscle M±σ
Glycogen[1]	16	113.9±56.4	420.8±139.2
Acid-soluble phosphate compounds[2]			
Acid-soluble total P	3	493.0±31.8	724.7±45.8
Inorganic P	3	103.8±23.9	62.6±11.5
Creatine P	3	129.8±15.0	216.4±12.6
Δ7P	3	119.3±8.0	233.8±24.9

1) mg. per 100 g. wet weight

2) μg. P per 100 mg. dry weight

M: arithmetical mean σ: standard deviation

The concentration of high energy phosphate compounds, *i.e.*, creatine phosphate and Δ7 phosphate was about two times higher in the white muscle than in the red muscle. Inorganic phosphate was rich in the red muscle, while total acid-soluble phosphate was rich in the white muscle.

TABLE II

Some Enzymatic Activities of the Red and the White Muscle of Rabbits

	Expt. No.	Red muscle M±σ	White muscle M±σ
Oxidation of pyruvate[1]	3	1.09±0.194	0.17±0.022
Endogenous respiration without substrate[1]	3	0.46±0.120	0.13±0.032
Oxidation of succinate[1]	6	2.61±0.618	0.42±0.203
Anaerobic glycolysis[2]	3	25.69±0.98	42.33±1.56
ATP-ase activity[3]	3	59.52±4.28	199.40±6.35

1) μl. of O_2 absorbed per 30 minutes per mg. wet weight

2) μg. of lactic acid produced per 30 minutes per mg. wet weight

3) μg. of inorganic phosphate produced per hour per mg. wet weight

M: arithmetical mean, σ: standard deviation

The Activity of Oxidation of Pyruvate—The oxidation of pyruvate and succinate proceeded lineally for 30 minutes by either the red or the white muscle. As shown in Table II, a marked difference was observed between the red and the white muscle, *i.e.*, the activity of pyruvate oxidation of the former homogenate was about 6 times higher than that of the latter. It was further observed that endogenous respiration without substrate was also higher in the red muscle than in the white.

The Activity of the Oxidation of Succinate—It was also found that the activity of succinic dehydrogenase present in the red muscle homogenate was about 6 times as high as that in the white muscle homogenate. These results indicated that the red muscle had higher activity of tricarboxylic acid cycle than the white muscle.

The Activity of Anaerobic Glycolysis—In contrast to the activity of tricarboxylic acid cycle, anaerobic glycolysis was lower in the red muscle, *i.e.*, the lactic acid produced for 30 minutes by the red muscle was about half as little as that produced by the white muscle.

The Adenosine Triphosphatase Activity—The amount of inorganic phosphate liberated by incubation of the muscle homogenate with adenosine triphosphate was represented in Table II. These results showed that the adenosine triphosphatase activity of the white muscle was about 3.3 times higher than that of the red.

DISCUSSION

Ranvier (*1*) revealed in 1880 that the white muscle contracted rapidly and the red muscle contracted slowly. Denny-Brown (*2*) demonstrated that the muscles of various animals were composed of red and white muscle fibers, and that the red muscle fibers contracted slowly and tonically and participated in postural adjustment, while the white muscle fibers contracted rapidly and strongly and took part in phasic movement.

In the previous histochemical studies (*7–9*), the author revealed that the red muscle fibers had more mitochondria and higher activity of respiratory enzymes than the white muscle fibers. The present investigation demonstrated that the activity of pyruvate oxidation and succinic dehydrogenase of the red muscle fibers of rabbits was six times as higher as that of the white muscle fibers. This result is compatible with that of the previous histochemical studies. The physiological feature of the red muscle fibers may be closely related to their high activity of oxidation. Since the red muscle fibers are continuously contracting, they may have higher oxidative recovery.

On the other hand, it seems reasonable that the white muscle fibers, which are rapid and strong in contraction are rich in $\Delta 7$ phosphate, creatine phosphate and glycogen, and have higher activity of anaerobic glycolysis and adenosine triphosphatase. The lower activity of tricarboxylic acid cycle in white muscle fibers may be explained by the fact that these fibers contract only in phasic movement and do not contract continuously. Therefore, they may have lower oxidative recovery.

It may be interesting to compare the present findings with the biochemical difference between the red and the white muscle of other animals reported by other investigators. The biochemical characteristics of fish red muscle were shown by Hotta *et al.* (*20*) and the difference between the red and the white muscle of sparrows was reported by Hayashi (*21*). The red muscle of fishes, sparrows and rabbits was revealed to have higher activity of respiratory enzymes than the white. Hotta showed in fish that the red muscle had higher activity of adenosine triphosphatase and contained more glycogen than the white, while the author demonstrated in rabbit that the white muscle had the higher activity and more glycogen than the red. According to Hayashi, the sparrow red muscle has higher activity of anaerobic glycolysis than its white muscle, and there is no difference in content of high energy phosphate compounds between the red and the white muscle.

Thus, many past papers showed that chemical constituents and metabolic activity of different striated muscles were variable, but there have no clear-cut reports explaining this variability. The previous histochemical and the present biochemical studies by the author suggest that this variability is due to the different ratio in content of red and white muscle fibers in each type of muscles.

SUMMARY

Employing the central part of *M. soleus* of rabbits as a red muscle fiber preparation and the posterior edge of *M. adductor magnus* as a white muscle fiber preparation, the quantitative differences of some labile constituents and some enzymatic activities between the red and the white muscle fibers were estimated and the following results were obtained.

1. The histochemical study of succinic dehydrogenase demonstrated that the red muscle fiber preparatoin used practically free of white muscle fiber and *vice versa.*
2. The white muscle fibers contained about 3.7 times as much glycogen as the red muscle fibers.
3. The white muscle fibers contained about 2 times as much creatine phosphate and $\Delta 7$ phosphate as the red muscle fibers. Total acid-soluble phosphate was rich in white muscle fibers, while inorganic phosphate was rich in red muscle fibers.
4. The activity of oxidation of succinate and pyruvate was about 6 times higher in the red muscle fibers than in the white muscle fibers.
5. The activity of anaerobic glycolysis was about 1.7 times higher in the white muscle fibers than in the red muscle fibers.
6. The activity of adenosine triphosphatase was about 3.3 times higher in the white muscle fibers than in the red muscle fibers.

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EFFECTS OF SOME COPPER COMPLEXES UPON THE OXIDATION OF 3,4-DIHYDROXYPHENYLALANINE AND URATE

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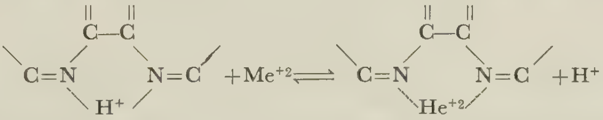
The metal chelates have been considered to afford clues to the behaviour of the active sites of metalloenzymes, and their catalytic nature has been investigated for many years from several sides (*1*). We have especially been interested in the behaviour of the complexes of copper with heterocyclic compounds as enzyme models, and have obtained certain results as published already (*2*). As chelating agents in the present work the organic unsaturated amines which possess the active functional group $=N-\overset{\|}{C}-\overset{\|}{C}-N=$ were chosen. It is to this group that such amines owe their capacity to form complex cations of the ring type chelate as they react with cupric ions:



We used dipyridyl, diquinolyl and *o*-phenanthrolines as examples of them.

As the first step toward the systematic study of their catalytic activity, the oxidation of DOPA by these copper chelates was investigated and the determinations of acidic ionization constants of these amines were also made. Previous work (*2b*) showed that some of these chelates could oxidize DOPA, that their catalytic activity to a certain extent parallel with the structural complexity, and further that this activity is much affected by the ratio of the metal ions to complexing agents at the time of mixing. It was also found previously that the cupric ions worked as an active catalyst upon the oxidation of urate in very alkaline solutions, and that the metal was activated when mixed with cyanide ions (*3*). In this case the copper cyanide was most active at the ratio 1 : 2. Now, we tried to find out among copper-amine chelates more potent catalysts of the urate oxidation in order to get further information on the urico-oxidase model and have found that some are superior in activity to copper cyanide.

We wish to make clear the interrelation of the oxidative activity, the stability constants and the redox potentials of these copper-amines and although some data have already been published in shorter forms, this report will deal with results of a wider study of the catalytic behaviour of the copper-amine chelates. The first step of getting information along the intended course is given here.

EXPERIMENTAL

Material Used—The chelating agents used in this experiment were as follows: dipyridyl, diquinolyl (cuproin), *o*-phenanthroline, 4,7-diphenyl-phenanthroline (bathophenanthroline), 2,9-dimethyl-phenanthroline (neocuproin), 2,9-dimethyl-4,7-diphenyl-phenanthroline (bathocuproin), and 2,9-diphenyl-4,7-dimethyl-phenanthroline (PMP). Bathocuproin and PMP were the generous gifts from Dr. G. F. Smith and other phenanthrolines were obtained from G. F. Smith Chemical Co. Dipyridyl and diquinolyl were purchased from Wako Pure Chemical Co. Water-insoluble materials were dissolved in alcohol. All inorganic chemicals used were of the analytical grade quality. Cupric sulphate was diluted before use from the stock solution of 0.1 *M*. Uric acid, from Merck, was dissolved in 0.1 *N* sodium hydroxide when used as the substrate in manometric determinations.

Manometric Determination—The oxygen uptake due to the oxidative breakdown of DOPA and urate by copper and its chelates was determined by using a Warburg manometer. Manometric flasks of around 13 ml. content each were shaken 120 times per minute in a water thermostat of 37.5°. Care was taken to prevent errors in manometric reading that might be caused by the alcoholic vapour, and the oxygen uptake in the first five minutes was canceled.

Determination of Acidic Ionization Constants of Monobasic Amines in 70 per cent dioxane—It is already known that a *o*-phenanthroline molecule combines with only one proton in acid solution (*4*). Dipyridyl and cuproin are also inferred to be in similar situations.

The acidic ionization constant of the monobasic amine is given by

$$K_{AH}=\frac{[H^+]\,[C_A-\{C_H-[H^+]\}]}{\{C_H-[H^+]\}},$$

where C_H is the concentration of the strong acid. In this experiment, C_A (amine) was about $2\times10^{-3}\,M$ and the concentration of nitric acid was $4.511\times10^{-3}\,M$ before the addition of potassium hydroxide. The content of the flasks titrated was 50 ml. and this was titrated with 0.4455 *N* potassium hyroxide. pH determinations were made by using a Toyorika pH meter, with an ordinary glass and a calomel electrode. The meter gave an accuracy of ±0.02 pH to standard buffers (pH 4.01, 6.99) before and after measurements in a water bath at 30°.

Since most amines are sparingly soluble in water, it was necessary to use a mixture of 70 per cent by volume of dioxane and water. Dioxane of analytical grade quality was from Wako and this was stored in a bottle over activated alumina (Merck after Brockman) and under nitrogen gas. In 70 per cent dioxane it was necessary to calibrate the pH meter, by titrating aqueous solution of a measured amount of nitric acid with dioxane and taking readings on the meter. The values obtained in this manner were checked against a titration of 70 per cent aqueous dioxane with 0.1 *N* nitric acid. The change in ionic strength throughout the titration and also the slight change in volume percentage can produce an error of ±0.03 in the correction factor. In this experiment, 0.6 was added to the pH read on the meter. The temperature of the solution to be measured was kept at 30±0.5° and a carbonate-free nitrogen atmosphere was maintained above the solution. Before and during determinations, the solution was swept with this gas, coming through two bottles of Fieser's solution (*5*), a bottle of saturated lead acetate, and a dessicator tube.

RESULTS

As shown in Fig. 1, Cu (II)-neocuproin was the most active in the mixing ratio of 1 : 1 or 1 : 1.5. However, when the mixing ratio reached 1 : 2, the Cu (II)-neocuproin became weaker and Cu (II)-*o*-phenanthroline grew stronger,

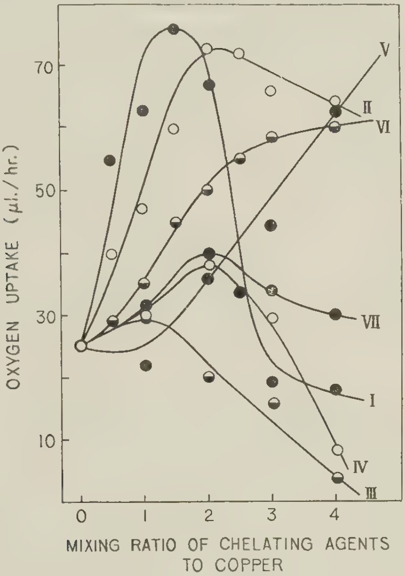


Fig. 1. Catalytic activity of copper chelates in oxidizing DOPA as affected by the mixing ratio of chelating agents to cupric sulphate (pH 7.8, 37.5°).

Each of the experimental flasks contained 0.2 ml. of 2×10^{-3} *M* cupric sulphate, a varying amount (0.1, 0.2, .. 0.6 ml.) of 2×10^{-3} *M* chelating agents, 0.5 ml. of *M*/15 phosphate buffer and a varying amount (1.6, ... 1.1 ml. according to the amount of chelating agent) of deionized water. The control had 0.2 ml. of 2×10^{-3} *M* cupric sulphate, 0.5 ml. of *M*/15 phosphate buffer and 1.7 ml. of deionized water. Each of the side arms of flasks contained 0.5 ml. of 10^{-2} *M* DOPA, and each of the wells, 0.2 ml. of 15 per cent potassium hydroxide.

Chelating agents themselves did not promote the autoxidation of DOPA in significant amount. The contents of the side arms were transferred into the main chambers when the flasks reached thermal equlibrium. The oxygen uptake in μl. per hour in experimental and control flasks is plotted against the concentration ratio of chelating agents to cupric sulphate.

Curve I:	Cu (II)-neocuproin.
Curve II:	Cu (II)-*o*-phenanthroline
Curve III:	Cu (II)-bathocuproin.
Curve IV:	Cu (II)-PMP.
Curve V:	Cu (II)-bathophenanthroline.
Curve VI:	Cu (II)-dipyridyl.
Curve VII:	Cu (II)-cuproin.

while Cu (II)-dipyridyl remained unchanged in the activity. Other Cu (II)-chelates were weak in their activity in these ratios. Beyond the ratio of 1 : 2, Cu (II)-neocuproin abruptly lost its activity and, at the ratio of 1 : 3 it worked as an inhibitor for the oxidation of DOPA. Cu (II)-*o*-phenanthroline gradually decreased its activity with the increase of the mixing ratio, though remained highly active even in the ratio of 1 : 4. The activity of Cu (II)-dipyridyl was enhanced step by step with the increase in the chelating agent, and the curve of the oxygen uptake by this chelate leveled off over the ratio of 1 : 3.

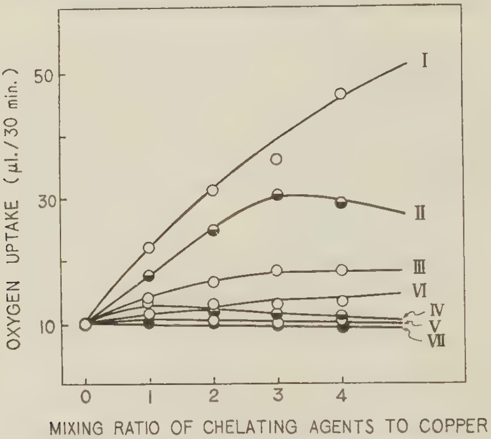


Fig. 2. Catalytic activity of copper chelates in oxidizing uric acid as affected by the mixing ratio of chelating agents to cupric sulphate (pH about 11.8, 37.5°).

Each of the experimental flasks contained 0.1 ml. of 10^{-4} *M* cupric sulphate, a varying amount (0, 0.1, 0.2,... 0.6 ml.) of 10^{-4} *M* chelating agents, and a varying amount (1.9,....1.3 ml.) of deionized water according to the amount of the chelating agents. Each of the side arms of the flasks contained 0.5 ml. of 50 mg. per cent uric acid, which was dissolved in 0.1 *N* sodium hydroxide. Total volume of the solution was 2.5 ml. Other conditions were the same as that in Fig. 1. Cupric ions and chelating agents did not show any oxidative phenomena when mixed with each other.

Curve I :	Cu (II)-phenanthroline.
Curve II :	Cu (II)-bathophenanthroline.
Curve III :	Cu (II)-neocuproin.
Curve IV :	Cu (II)-bathocuproin.
Curve V :	Cu (II)-PMP.
Curve VI :	Cu (II)-dipyridyl.
Curve VII :	Cu (II)-cuproin.

Some weak catalysts, such as Cu (II)-bathocuproin, -PMP, and -cuproin were converted to inhibitors as the ratio exceeded 1 : 3 or 1 : 4, but that of bathophenanthroline followed a different course. Cu (II)-bathophenanthroline behaved in a peculiar way. It rather inhibited the oxidation of DOPA at the ratio of 1 : 1, but as the ratio increased, it gradually displayed oxidative power in a like manner as did Cu (II)-dipyridyl, and remained still highly active even at 1 : 6.

As preliminary work revealed to us that uric acid would also be oxidized by Cu (II)-phenanthroline, some manometric experiments were carried out with this acid in the manner similar to the above. Fig. 2 tells us that Cu (II)-phenanthroline was the first, Cu (II)-bathophenanthroline the next, and Cu (II)-neocuproin the third in the order of the catalytic strength. Other copper chelates were very weak in activity to catalyze the oxidation of uric acid. In the case of Cu (II)-phenanthroline the activity did not come to the maximum until the mixing ratio reached 1 : 18, and beyond this ratio it remained constant, whereas Cu (II)-bathophenanthroline showed the highest activity at the ratio of 1 : 3, and Cu (II)-dipyridyl was a weak catalyst. The other three, Cu (II)-bathocuproin, -PMP, and -cuproin were rather inhibitory. Thus copper chelates behaved differently toward uric acid as compared with the case of DOPA.

DISCUSSION

It has been shown in Fig. 1 that the mixing ratio of cupric ions to amines greatly affects the oxidation of DOPA by the copper chelates. When the ratio was 1 : 1, the strength of the activating power of ligands on the copper was in the order : neocuproin > *o*-phenanthroline > bathocuproin ≧ PMP > bathophenanthroline, and dipyridyl > diquinolyl. As will be seen in Table I, we carried out the determination of the acidic ionization constants of these amines in 70 per cent dioxane solution. The order of the pK values (negative logarithms of the acidic ionization constants) was as follows: neocuproin > *o*-phenanthroline > bathocuproin > PMP > bathophenanthroline > dipyridyl > diquinolyl. It might be generally said from these data that the higher the pK of an amine, the stronger is the catalytic activity of its copper chelates. This holds good only when the mixing ratio is 1 : 1 or up to 1 : 1.5.

TABLE I

Acidic Ionization Constants of Mono-basic Amines in 70 per cent Dioxane Solution.

	pK
neocuproin	5.24
o-phenanthroline	4.87
bathocuproin	4.80
PMP	4.50
bathophenanthroline	4.26
dipyridyl	3.77
diquinolyl	3.28

When the mixing ratio is above 1:1, certain other factors would also play some roles in the oxidative reaction. Cu (II)-neocuproin showed the highest activity at 1:1.5, but at 1:2, the activity declined. The methyl groups of neocuproin would serve as an electron-releasing factor, and would make the molecule more basic. This would be very useful for the oxidation of DOPA, for, with their aid, the turnover of valence state of the copper would be facilitated to oxidize the substrate. But, if mixed in the ratio 1:2, four methyl groups around the metal atom would hinder the reaction between the substrate and the metal. The increase of the mixing ratio of neocuproin resulted to some extent in the development of the specific colour of Cu (I)-neocuproin; the turnover of the valence state became difficult in the solution containing large amounts of the ligand.

The amines form complexes of high stability with copper* and in the ratio 1:2, the complex: copper $(\text{amine})_2$ would predominate in the pH range employed in this experiment. The coordinating bonds of the central atom will fully be saturated in this ratio. When DOPA is oxidized, it should combine, if temporarily, with the central atom, and consequently competition would occur between ligands and the substrate molecules. However, as the free metal ions would be decreased in the solution with the further addition of the ligand, a coordination in the copper-amine chelates would be well established. In addition to this, steric configuration of such large groups as methyl or phenyl in 2- and 9- positions would hinder the access of the substrate. The occurrence of steric hindrance in Cu (II)-neocuproin, in the ratio of 1:2, would be known from the formation curves of the chelate, although in 70 per cent dioxane solution*. The conversion of cupric forms to cuprous, stability constants and other factors would thus greatly affect the catalytic activity of these copper chelates. In a similar manner, many copper chelates would inhibit in the excess of the ligands.

Bathophenanthroline (Curve V, Fig. 1) acted in a peculiar way when mixed with cupric ions. In spite of its rather inhibitory nature in the ratio 1:1, it gradually aquired oxidative power with the increase of the ratio, and remained very active even at 1:6. This powerful activity of the phenanthroline in excess amounts remained unexplicable. A similar relation would hold in the case of Cu (II)-dipyridyl (Curve VI).

It was revealed that the urate suffers oxidation by Cu (II)-*o*-phenanthroline in highly alkaline solulions. Although many of the copper-phenanthrolines were quite effective upon DOPA, Cu (II)-neocuproin showed but a weak activity in the oxidation of the urate. Cu (II)-*o*-phenanthroline was the most active catalyst, but it did not attain the maximal activity, until the concentration of the ligand reached 15 times that of the metal ions. Both Cu (II)-neocuproin and -bathophenanthroline had the optimal mixing ratio at 1:3. In the oxidation of urate, we could divide the unsaturated amines into three groups according to the order of their activities: 1) *o*-phenanthroline>bathophenanthroline, 2) neocuproin>bathocuproin>PMP, and 3) dipyridyl>diquinolyl. In this case, also, the ligands in each group follow the order of their pK values.

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* unpublished data

It is interesting to see that the substitution in 2- and 9- positions of the molecule, whether by methyl or phenyl, resulted in the inhibition of the oxidation of urate by the central metal atom contrary to the case of DOPA oxidation. Thus, the activity of Cu (II)-neocuproin was situated below that of Cu (II)-phenanthroline, although pK of the neocuproin is greater than that of *o*-phenanthroline. Cu (II)-bathocuproin and -PMP were much weaker and rather inhibitory when a large excess of the ligands was present. The access of the substrate to the metal would be hindered by certain- 2- and 9-substituting groups, in a different manner from that in the oxidation of DOPA. The necessary configurations to oxidize the substrates around the metal should be quite different according to the type of the substrate. So far as the oxidation of urate catalyzed by *o*-phenanthroline derivatives is concerned, it would at least be necessary to avoid substituting 2- and 9-positions with methyl or phenyl group, so as to let the substrate come easily into contact with the copper. The space will be necessary to be as large as possible around the central atom in order to get the urate near to the metal. A narrower space will suffice in the oxidation of DOPA.

As described above, the valence state of the copper was to a certain degree reduced to the cuprous state after the addition of cuproin or bathocuproin or neocuproin. These ligands cannot be employed for determining the valence state of the central metal ion in the chelate compound or metalloenzymes. From this point of view, one may cast some doubt on the validity of the view taken by Kertez (*6*), who claimed the presence of cuprous copper in his preparation of polyphenoloxidase, based on the observation that the enzyme solution, when treated with cuproin, gradually developed a purple colour.

These results would contribute to a further project of synthesizing more potent copper catalysts for the oxidation of DOPA and urate, and would also serve as a key to investigate the evolution of enzymes or the active site of the enzymic protein from the viewpoint of the enzyme model.

SUMMARY

1. Acidic ionization constants of some unsaturated amines were determined in 70 per cent dioxane solution. The order of pK values was as follows: neocuproin > *o*-phenanthroline > bathocuproin > 2,9-diphenyl-4,7-dimethyl-phenanthroline (PMP) > bathophenanthroline > dipyridyl > diquinolyl.

2. The acceleratory effect of cupric ions upon the oxidation of DOPA was activated when some unsaturated amines were added. When the mixing ratio of copper to amines was 1 : 1, the order of the activity coincided with that of their pK values. Beyond this ratio, some of them decreased more or less in activity while some enhanced further the catalytic activity of the cupric ions.

3. The catalytic power of cupric ions upon the oxidation of urate was

also activated if some amines were added. The order of the activity was as follows: 1) *o*-phenanthroline>bathophenanthroline, 2) neocuproin>bathocuproin>PMP, 3) dipyridyl>diquinolyl. These orders in each group coincide with those of their pK values. Discussions were made concerning the catalytic activity of the amines referring to their steric configurations.

The author is greatly indebted to Prof. Adrien Albert of the Australian National University who has read the manuscript and made valuable comments on it.

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STUDIES ON THE METABOLISM OF DIHYDROXYFUMARATE, HYDROXYPYRUVATE AND THEIR RELATED COMPOUNDS

I. ENZYMATIC FORMATION OF XYLULOSE IN RAT LIVER*

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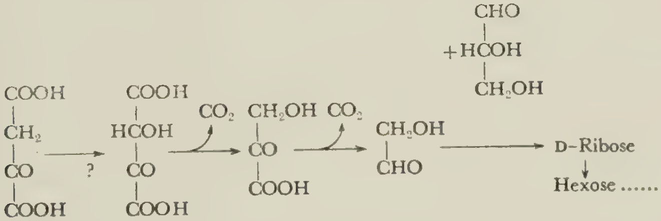
Dihydroxyfumaric acid has been found to have biological significance since Neuberg (*2*) observed that DHF was fermented by yeast. In 1930, Stepanow *et al.* (*3*) reported the formation of HPA and DHF from glyoxylate in yeast. It was also found that the content of glycogen was increased in muscle on incubation with DHF (*4*). In recent years, Akabori *et al.* (*5*) presented the following sequence of enzyme reactions as a new pathway for glyconeogenesis, based on the observation suggesting the formation of a pentose (or a pentose phosphate) on addition of DHF and glyceraldehyde (or fructose-1,6-diphosphate and aldolase as a source of glyceraldehyde-3-phosphate)



$$\begin{matrix}\mathrm{COOH}\\|\\\mathrm{CH_2}\\|\\\mathrm{CO}\\|\\\mathrm{COOH}\end{matrix} \xrightarrow[?]{} \begin{matrix}\mathrm{COOH}\\|\\\mathrm{HCOH}\\|\\\mathrm{CO}\\|\\\mathrm{COOH}\end{matrix} \xrightarrow{\mathrm{CO_2}} \begin{matrix}\mathrm{CH_2OH}\\|\\\mathrm{CO}\\|\\\mathrm{COOH}\end{matrix} \xrightarrow{\mathrm{CO_2}} \begin{matrix}\mathrm{CH_2OH}\\|\\\mathrm{CHO}\end{matrix} \xrightarrow{+\begin{matrix}\mathrm{CHO}\\|\\\mathrm{HCOH}\\|\\\mathrm{CH_2OH}\end{matrix}} \begin{matrix}\text{D-Ribose}\\\downarrow\\\text{Hexose}\end{matrix} \;\ldots\ldots\; (1)$$

in rabbit muscle extract. But the product was identified only by paper chromatography and was assumed to be D-ribose. No further confirmation was made. It was reported (*6*) that the step of the decarboxylation of DHF was accelerated by metal ions such as Co^{++}, Mn^{++}, or Mg^{++} in the presence of rabbit muscle extract. This decarboxylation reaction was presumed to be non-enzymatic. Hitherto any enzyme which decarboxylates DHF has not yet been obtained from animal tissues.

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* This work was presented at the 30th Annual Meeting of the Japanese Biochemical Society in Kyoto, July, 1957, at the 31st Annual Meeting of the same society in Sapporo, July, 1958 and at the 11th Symposia on Enzyme Chemistry in Kyoto, July, 1959.

Preliminary report of this work has already been published (*1*).

The following abbreviations are used: dihydroxyfumarate, DHF; hydroxypyruvate, HPA; tartronic acid semialdehyde, TAS; Glyceraldehyde, Gla; xylulose, Xu; transketolase, TK; Oxaloacetate, OAA.

HPA has now been proved to be an effective substrate for various enzymes such as L-lactic dehydrogenases of muscle (*7*) and yeast (*8*), D-glyceric dehydrogenase (HPA reductase) (*9*) in higher plants, carboxylase of yeast (*10*), and for transketolases of spinach (*11*) and yeast (*12*). The possible biological importance of HPA was also shown concerning the metabolism of serine (*13*, *14*, *15*). The natural occurrence of HPA in animal tissues has been also reported (*16*). However, it has not yet been reported that HPA can be a substrate for TK of animal sources. TAS has not been found to be a substrate for any enzyme.

In the present study, the formation of pentose was observed from either DHF, TAS, or HPA, and Gla with purified rat liver TK and the product was confirmed to be Xu. The decarboxylation of DHF by partially purified enzyme from rat liver was also investigated.

MATERIALS AND METHODS

Materials—DHF was synthesized from tartaric acid by oxidation according to the modified method of Hartree (17).

Lithium hydroxypyruvate was prepared according to the method described by Dickens *et al.* (*18*) and recrystallized once from water.

TABLE I

Properties of TAS, DHF and HPA

	TAS	DHF	HPA
Decolorizing activity of 2,6-dichlorophenolindophenol	(+)	rapid (+)	weak (±)
Schiff's reaction	(+)	(−)	(−)
Formation of 2,4-dinitrophenylhydrazone	bis (+)	mono (+)	mono (+)
Formation of *p*-nitrophenylhydrazone	react very rapidly[1]		react slowly
Formation of phenylosazone	(+)[2]	(−)	(−)
Iodine consumption	(−)	discolored rapidly	(−)
Spontaneous decarboxylation (in 0.25 *M* phosphate buffer pH 6.2 at 30°)	(+) not accelerated by Mn^{++}	(+) accelerated by Mn^{++}	(−)

1) mp. (decomp.) 233–236°. mp. reported, 240°. (*21*).
2) mp. (decomp.) 210–215°. mp. reported, 218–220°. (*18*).

Potassium salt of tartronic acid semialdehyde was made from DHF by decarboxylation. Two g. of DHF was suspended in 20 ml. of distilled water with 3 μM of Mn^{++} and decarboxylated by heating to 40° in the presence of coal gas. The reaction mixture was evaporated to a thick syrup at reduced pressure in coal gas. Slightly yellowish thick syrup was dissolved in 30 ml. of absolute ethanol and neutralized with KOH dissolved in absolute ethanol with continuous stirring. Precipitate was collected, filtered and dried in an evacuated desiccator. Very hygroscopic white powder was obtained. The product thus obtained had the properties identical with those of tartronic acid semialdehyde described by Fischer *et al.* (*19*). This substance gave one spot on paper chromatogram, using two solvent systems (ethanol-acetic acid-water, 100 : 1 : 25, and butanol-ethanol-water, 4 : 1 : 2). Properties of this compound are shown in Table I in comparison with those of DHF and HPA.

Xu was synthesized from xylose according to the method of Schmidt *et al.* (*20*).

Gla and thiamine pyrophosphate were obtained from commercial sources.

Enzymes—TK was prepared from rat liver according to the method described by Horecker *et al.* (*11*). The second and third acetone fractions were combined and dissolved in cold water and used as a purified TK. This preparation was purified 15 folds from acetone powder extract.

DHF decarboxylase was purified from rat liver.

Analytical Methods—The determination of Xu was made on the eluate from the paper chromatogram by the orcinol test (*22*), the cysteine carbazole reaction (*23*) and by the resorcinol reaction (*24*).

Paper chromatography of the sugar was carried out on Toyo Filter Paper No. 50 with 80 per cent phenol or butanol-ethanol-water (52 : 32 : 16) by the ascending method. The chromatograms were sprayed first with TCA-orcinol (*25*) and heated at 100° for 5 minutes, and then oversprayed with aniline phosphate reagent (*26*).

TAS was determined as 2,4-dinitrophenylhydrazine derivative in the presence of 0.02 *M* EDTA which protects further decarboxylation of DHF. 2,4-Dinitrophenylhydrazine derivatives were extracted and separated on the paper chromatogram according to the method of Cavallini (*27*) or Kun (*28*). TAS derivative was extracted from the the paper chromatogram with 1 *N* NaOH and the absorption spectrum was determined using a Beckman spectrophotometer.

For the quantitative determination absorbance was measured at 545 mμ for the 2,4-dinitrophenylhydrazine derivative of TAS.

Total aldehyde formed from DHF was determined with Schiff's reagent also in the presence of 0.02 *M* EDTA. 1 ml. of this reagent was added to 1 ml. of the reaction mixture deproteinized with TCA to the final concentration of 2.5 per cent. After standing at room temperature for one hour absorbance at 560 mμ was measured against a reagent blank.

The concentration of protein in the enzyme solution was determined by ultraviolet absorption at 280 mμ and 260 mμ according to Warburg and Christian (*29*).

Assay Methods—Standard assay system for TK contained 75 μM of Tris buffer (pH 7.6), 30 μM of $MgCl_2$, 1 μM of cocarboxylase, 3 μM of cysteine hydrochloride, 15 μM of Gla and 15 μM of either DHF, TAS or HPA, and the enzyme. The total volume was 3.0 ml. Incubation was carried out anaerobically at 30° for 1.5 hours.

DHF decarboxylase activity was determined by measuring carbon dioxide evolution from DHF by the enzyme. The reaction mixture consisted of 75 μM of acetate buffer (pH 5.0), 4 μM of $MnCl_2$, and 30 μM of DHF in a final volume of 3.0 ml. DHF was brought to pH 5.0 with KOH before use. DHF was tipped from the sidearm of a Warburg flask after the preincubation for 5 minutes at 30°. At the end of the incubation for 60 minutes, 0.2 ml. of 30 per cent of $HClO_4$ or 0.2 ml. of 38 per cent TCA was tipped in from a second sidearm. The rate of spontaneous decarboxylation of DHF was determined by measuring the CO_2 evolution in a control flask containing the same content as mentioned above except for boiled enzyme. Non-enzymatic decarboxylation was also measured using another flask containing no enzyme. However, the difference of the rate of decarboxylation between the two controls decreased as the enzyme was further purified. Therefore, the difference of the amount of CO_2 evolved between the native enzyme and the boiled enzyme was considered to be due to the enzymatic decarboxylation. Incubation was carried out in N_2 which was absolutely free from O_2. Trace of O_2 was removed by the Vanadine method (*30*).

RESULTS

Xu Formation from DHF, TAS, HPA and Gla by Purified Enzyme

Paper Partition Chromatography—Formation of Xu was demonstrated by paper chromatography. The spots showed characteristic color of Xu as shown in Table II, while the control in which DHF, TAS or HPA was omitted showed no color development except for brownish spot characteristic for Gla. Other pentoses were not found.

TABLE II

Paper Chromatography of Reaction Product

	Solvent: 80% phenol R_f	Solvent: BuOH-EtOH-water R_f	Color developed	Color after aniline phosphate
Reaction product	0.43	0.57	steel grey	greyish purple
Authentic Xu	0.43	0.57	,,	,,

The assay system is given in the text under 'method'. After deproteinization with 0.1 ml. of 60% $HClO_4$ the mixture was centrifuged and the supernatant solution was neutralized with 4 *N* KOH to pH 6.0. $KClO_4$ was removed by centrifugation. The solution was deionized by passage through a column of Amberlite IR 120 (H^+) and IR 4B (OH^-) mixed bed resin (1.5×12 cm.). The effluent and washings were taken and dried at reduced pressure in the stream of coal gas. The residue was extracted with 0.5 ml. of ethanol and applied to paper chromatography.

Orcinol Reaction—The spectrum of the product of the orcinol reaction has two absorption maxima at 670 mμ and 540 mμ as shown in Fig. 1, and the absorption ratio $E_{540m\mu}/E_{670m\mu}$ was 0.33~0.38 (*26*, *31*) which is characteristic for Xu, while 0.8~1.0 was the ratio for ribulose.

Resorcinol Reaction—The spectrum of the product of the resorcinol test was also identical with that of authentic Xu as shown in Fig. 2.

Cysteine Carbazole Reaction—The product of the cysteine carbazole reaction showed an absorption maximum at 540 mμ which is characteristic for ketopentoses and the color development reached the maximum after two hours. This rate of the cysteine carbazole reaction is characteristic for Xu and differentiates it from ribulose for which the color is completely developed in less than ten minutes (Fig. 3).

Column Chromatography—The separation of the products was attempted by column chromatography using Dowex-1-borate according to the procedure of Khym and Zill (*32*) and Lampen (*33*).

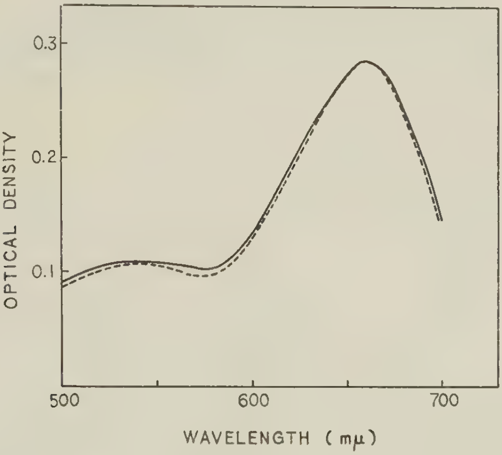


FIG. 1. Absorption spectrum of the product of orcinol reaction. —— product, ---- authentic.

Stability of the Product to Bromine—For the oxidation by bromine, 1 ml. of the sample, 0.25 ml. of 1.2 per cent bromine solution and 20 mg. of $BaCO_3$ were incubated at 20° for 1 hour. The excess bromine was removed by gassing with nitrogen, and the resultant sample was then filtered for assay of residual pentose. The value for orcinol was decreased to 80 per cent after the oxidation. This stability is characteristic for ketoses and differentiates the product from aldose such as ribose.

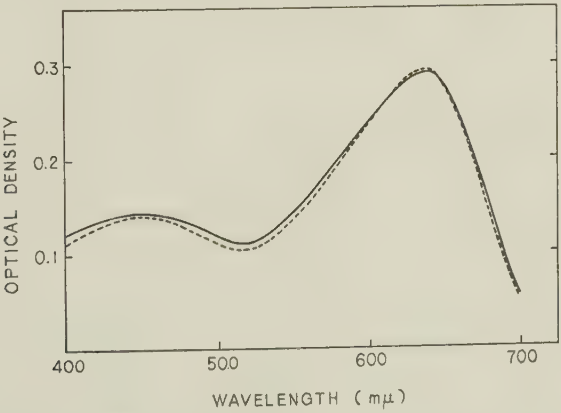


FIG. 2. Absorption spectrum of the product of resorcinol reaction. —— product, ---- authentic.

Stability of the Product to Alkali—Samples were incubated at 25° in 1 *N* KOH and an aliquot was removed for orcinol assay. The control samples (zero time) received equivalent amount of alkali after addition of $FeCl_3$-HCl

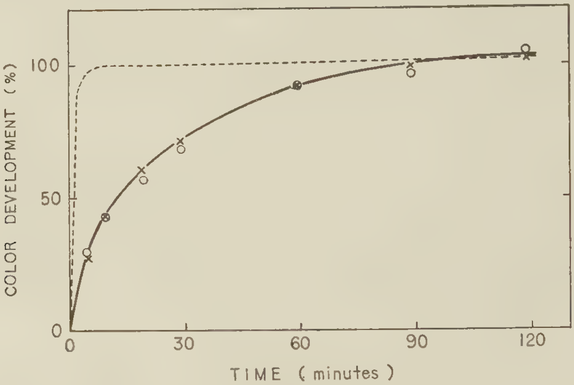


FIG. 3. Time course of color development of cysteine carbazole reaction of the product.
—○— product, —×— authentic Xu, ---- ribulose.

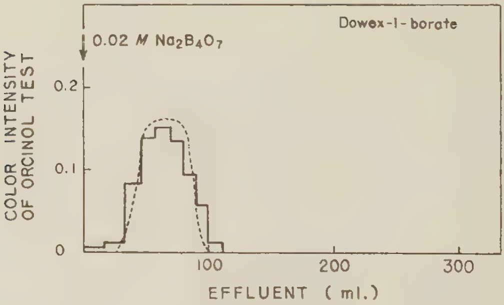


FIG. 4. Column chromatography of the reaction product. Incubation mixture for the large scale experiment contained 250 μM of Tris buffer (pH 7.6), 150 μM of $MgCl_2$, 750 μg. of cocarboxylase, 1.5 mg. of cysteine hydrochloride, 50 μM of Gla, 50 μM of DHF and the enzyme. Total volume was 10 ml. Gas phase was air. The mixture was incubated for 2.5 hours at 30° and deproteinized with 0.5 ml. of 60% $HClO_4$ and neutralized with 4 *N* KOH to pH 6.0. After removal of $KClO_4$, the reaction mixture was deionized by passing through a mixed bed resin (Amberlite IR 120 (H^+)+IR 4B (OH^-)). The effluent was concentrated and dissolved in 5 ml. of 0.01 *M* borate. The solution was adsorbed on a Dowex-1 column and eluted with 0.02 *M* sodium borate. The fractions were passed through a column of Amberlite IR 120 (H^+) to convert the borate to the free acid and the solutions were evaporated to dryness at reduced pressure. Boric acid was removed as methanol borate and the final residue was dissolved in water or ethanol for analysis. —— product, ---- authentic Xu.

reagent. The decrease in orcinol color of the product on alkali treatment for 10 minutes was 85 per cent, which indicated the product to be alkali labile, in comparison with the ribose which lost only 20 per cent of the color.

Formation of Xu—Xu was formed from either DHF, TAS or HPA, and Gla as shown in Table III. The amount of Xu formed from DHF, which requires two steps of decarboxylation to give "active glycolaldehyde", was two to three times as much as that from HPA. This point will be discussed later.

Decarboxylation of DHF

Purification of Enzyme—Three g. of acetone powder of rat liver were extracted with 8 volumes of ice cold 0.001 *M* potassium phosphate buffer (pH 7.8) for 15 minutes at 38° with occasinol stirring. The clear supernatant was obtained by centrifugation at 5000 r.p.m. for 10 minutes.

TABLE III

Formation of Xu from DHF, TAS and HPA

Substrate	DHF	TAS	HPA
Xu formed (μM)	0.53	0.51	0.18

Conditions were the same as described under 'method'.

Fractionation with Ammonium Sulfate—To the supernatant solution $(NH_4)_2SO_4$ solution (saturated at 2°) was added slowly with stirring to give 30 per cent saturation. The precipitated protein was removed by centrifugation. To the supernatant solution $(NH_4)_2SO_4$ was added to bring the concentration to 60 per cent saturation. The precipitate was centrifuged after 30 minutes, and dissolved in 15 ml. of 0.01 *M* potassium phosphate buffer (pH 7.4).

Methanol Fractionation—After the pH of the solution was adjusted to pH 7.5 with 2 *N* NH_4OH, enough cold water was added to bring the protein concentration to 10 mg. per ml. Sufficient methanol previously cooled to −20° in a dry ice acetone bath were added to the above enzyme solution to 30 per cent in about 30 seconds, and then the solution was cooled to −13° to −16° for 1 to 2 minutes. The precipitate was removed by centrifugation for 2 minutes at −10° and the supernatant solution was treated as described above with cold methanol to a final concentration of 50 per cent. The precipitate was suspended in 6 to 7 ml. of 0.01 *M* potassium phosphate buffer (pH 7.4) and was dialysed overnight against 0.005 *M* potassium phosphate buffer (pH 5.8) at 1° to 2°. The insoluble residue was then centrifuged and discarded. The enzyme was purified 13 folds from acetone powder extract, as shown in Table IV.

Decarboxylation of DHF—Vigorous decarboxylation (Fig. 5) occurred immediately after addition of DHF to the enzyme and continued at a linear rate at least for 1 hour, while spontaneous decarboxylation also took place when metal ion was present. The amount of CO_2 evolution accelerated by the enzyme was twice that of the spontaneous one. TAS was decarboxylated spontaneously, but no increase of decarboxylation was observed with the enzyme and the degree of decarboxylation was less than the spontaneous decarboxylation from DHF. HPA was not decarboxylated under the same condition either enzymatically or spontaneously.

TABLE IV

Purification of DHF Decarboxylase

Fraction	Total unit[1]	Specific activity[1]
Acetone powder extract	13.6	0.39
Ammonium sulfate fraction (30–60%)	10.1	0.97
Methanol fraction (30–50%)	5.2	4.60

1) μM of CO_2 per mg. of protein per 60 minutes.

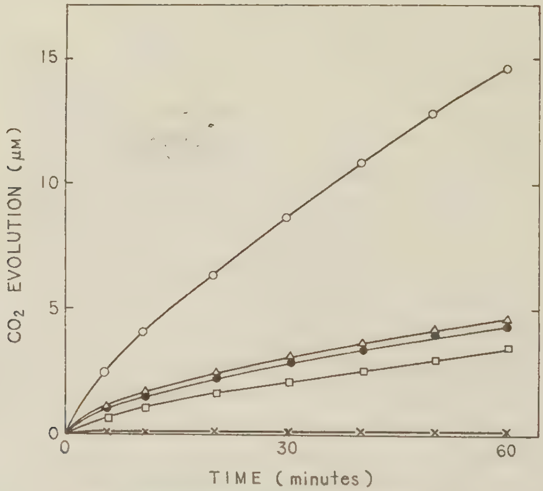


FIG. 5. Decarboxylation of DHF. Conditions were the same as described under 'method'. Protein content, 1.7 mg.
—○— complete system, —△— with boiled enzyme,
—●— without enzyme, —□— TAS, —×— HPA

Effect of pH—The decarboxylation reaction proceeded at a maximal velocity at pH 5.0 (Fig. 6.). Above and below this pH the rate of reaction declined, but was still active at pH 7.2, at which TK had an activity enough to form Xu.

Metal Requirement—The requirement for divalent metal ions was evident (Table V). Mn^{++}, which was the most effective ion tested, showed the maximum activity at 1.3×10^{-3} *M* (Fig. 7). Co^{++} has almost the same effect as Mn^{++}. Mg^{++} was less effective.

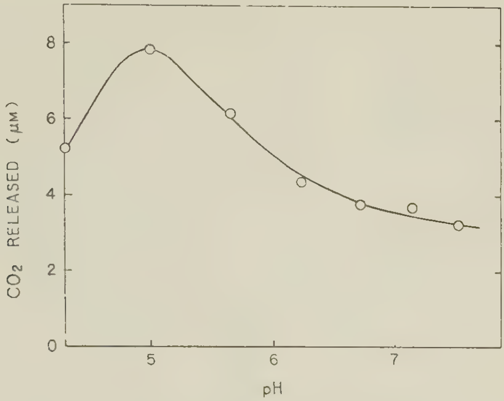


FIG. 6. Effect of pH on DHF decarboxylase.
pH 4.4~5.6 0.025 *M* Acetate buffer
pH 6.2~7.5 0.025 *M* Phosphate buffer
pH 7.2 0.025 *M* Tris buffer

Reaction Product—The chromatography of 2.4-dinitrophenylhydrazine derivative of the reaction product is shown in Fig. 8. The main product was bis-2,4-dinitrophenylhydrazone (osazone) and its R_f was identical with that of bis-2,4-dinitrophenylhydrazone of synthetic TAS. Trace amount of hydrazone identical with HPA monohydrazone was also observed.

TABLE V

Metal Requirement for DHF Decarboxylation

Metal	Concentration	CO_2 evolution (μM)
None	—	1.1
Mn^{++}	1.3×10^{-3} *M*	9.0
Co^{++}	,,	8.9
Fe^{++}	,,	6.0
Mg^{++}	,,	5.5

Conditions were the same as described under 'method'. Enzyme protein, 1.8 mg.

Stoichiometry—The quantitative relationship of the products is shown in Table VI.

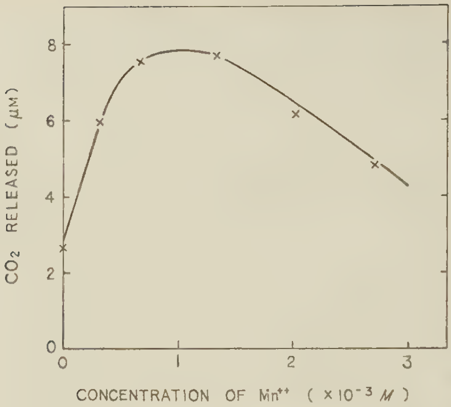


FIG. 7. Effect of Mn^{++} concentration.

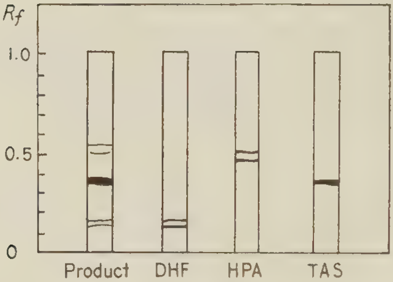


FIG. 8. Chromatogram of decarboxylation product.

TABLE VI

Decarboxylation of DHF and TAS Formation

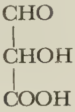
	Enzymatic	With boiled enzyme	Spontaneous
CO_2 released (μM)	13.5	4.2	4.1
TAS formed (μM)	11.2	3.7	3.6

TAS was determined as bis-2,4-dinitrophenylhydrazone.

DISCUSSION

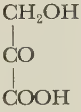
Horecker *et al.* (*11*) reported the formation of ketopentose, which was presumed to be ribulose, from HPA and Gla by purified spinach TK. De la Haba *et al.* (*12*) also observed the formation of ketopentose by crystalline yeast TK and the product was identified to be ribulose. Uehara (*34*) reported the formation of pentose from DHF and Gla or fructose-1,6-diphosphate and aldolase as a source of Gla-3-phosphate in rabbit muscle extract. The product was presumed to be D-ribose. Recently evidences have been obtained that Xu-5-phosphate, and not ribulose-5-phosphate, was the true substrate for yeast (*35*) and liver (*36*) TK. However, their experiments were not concerned with Xu formation from either HPA or other related compounds. In the present study it was confirmed that Xu was formed from either DHF, TAS or HPA, and Gla by purified rat liver TK. The amount of Xu formed from HPA which is a direct precursor of "active glycolaldehyde" was 1/2 to 1/3 of that from DHF. On the contrary, with the purified yeast TK, the amount of Xu formed from HPA was three times as much as that from DHF*. It was reported that both carboxylase and TK might be required for the formation of erythrulose or ketopentose, which was shown in yeast to be a result of vigorous decarboxylation of HPA and following acyloin condensation with a suitable acceptor aldehyde (*37*). Accordingly, if the decarboxylating enzyme for TAS is richer than that for HPA in liver TK prepared in the present study, Xu formation from TAS may be greater than that from HPA. For this reason, HPA or TAS decarboxylating enzyme and interactions between these enzymes and TK are now under investigation, which may clarify that cause of the less formation of Xu from HPA. It is probable that by the addition of carboxylase or similar enzyme the activity of TK for HPA will be enhanced. Since DHF and TAS gave almost the same amount of Xu, and DHF was proved to be decarboxylated to give TAS, Xu may be formed from DHF *via* TAS.

Obviously DHF was decarboxylated to give a substrate for TK either enzymatically or spontaneously. Three possible structures may be assumed for the C_3 compound of this decarboxylation product, *i.e.* TAS (I), HPA (II) and dihydroxyacrylic acid (III).



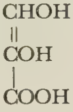
$\begin{array}{l} CHO \\ | \\ CHOH \\ | \\ COOH \end{array}$

(I)



$\begin{array}{l} CH_2OH \\ | \\ CO \\ | \\ COOH \end{array}$

(II)



$\begin{array}{l} CHOH \\ \| \\ COH \\ | \\ COOH \end{array}$

(III)

Tautomerism between these three are also possible. Uehara (*38*) presumed HPA to be the product of decarboxylation. Sasagawa (*39*) reported the enzymatic decarboxylation of DHF by the extract of avain tubercle bacilli and found HPA by paper chromatography as one of the products. Kun (*40*) also reported the non-enzymatic decarboxylation of DHF by the action of metal ion and found HPA as a product. In another paper (*41*) the same author mentioned about bis-2,4-dinitrophenylhydrazone of HPA. However, in the present study no bis-2,4-dinitrophenylhydrazone was obtained from synthetic HPA. Dickens (*18*) also stated that no phenylosazone was obtained from HPA, but only monohydrazone. Therefore in the present experiment it can be concluded that TAS, which forms bis-2,4-dinitrophenylhydrazone, is the major product of decarboxylation of DHF either enzymatically or spotaneously, though only a small amount of HPA was formed.

Studies of the non-enzymatic decarboxylation of OAA have been made by many workers (*42*, *43*) and it was presumed that the metal-catalyzed decarboxylation of OAA proceeds *via* a metal complex of the enol type of pyruvate. By analogy with metal-catalyzed decarboxylation of DHF, dihydroxyacrylic acid may be a possible direct product. On the other hand, it is not yet clarified whether keto form, *i.e.* oxaloglycolate or dienol form, *i.e.* DHF is decarboxylated directly, since it is known that DHF is a relatively stable dienol. A solution of DHF exhibits a strong absorption band at 290 mμ, characteristic of its dienol structure. However, the absorbance at 290 mμ diminished rapidly when Mn^{++} was added to the solution, condition of which was the same as the assay system. This evidence and TAS formation as a product may suggest the possibility of decarboxylation of carboxyl group next to the carbonyl group of keto form.

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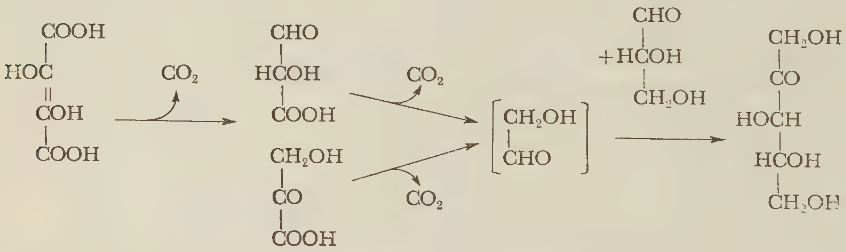
* unpublished data.

It was considered that DHF might be decarboxylated by OAA carboxylase (*44*), malic enzyme (*45*) or OAA decarboxylase (*46*), optimal pH of which were 6.2, 4.4 and 7.5, respectively. However, at pH 5.0 OAA was not decarboxylated by this enzyme at all. Therefore this enzyme was proved to be essentially different from OAA decarboxylating enzymes. To clarify the mechanism of the enzyme action further purification will be required.

In conclusion, it is now possible to formulate a sequence of reactions leading from DHF to pentose as follows, instead of the scheme (I) which has been presented by Akabori *et al.*.



SUMMARY

1. Xu was identified to be the reaction product from either DHF, HPA or TAS, and Gla by purified rat liver transketolase.

2. The amounts of Xu formed from HPA was less than that from DHF, which requires two steps of decarboxylation to give "active glycolaldehyde", Possible explanation for this was discussed.

3. DHF was found to be decarboxylated enzymatically. The decarboxylase was purified 13-fold from acetone powder of rat liver. It required divalent cations such as Mn^{++}, Co^{++} for activation. pH optimum was pH 5.0. This enzyme was distinct from oxaloacetic acid decarboxylating enzymes and exhibited no activity with OAA at pH 5.0.

4. The main product of decarboxylation was identified to be TAS.

The author wishes to express her gratitude to Dr. K. Kuratomi for his kind guidance and encouragement throughout the investigation and to Prof. T. Sekine and Prof. S. Akabori for their continuous interest.

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A LIMITED PROTEOLYSIS OF NATIVE OVALBUMIN BY BACTERIAL PROTEINASE

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An interesting enzymic modification of ovalbumin has been described by Linderstrøm-Lang and Ottesen (*1*). They obtained a new protein by the action of subtilisin on ovalbumin. The new protein crystallized in the form of plates instead of needles characteristic to ovalbumin and for this reason the newly formed protein has been named plakalbumin (*2*). It was later reported that native ovalbumin incubated for 5 hours at 30° with an enzyme preparation from *Bacillus subtilis* gave plakalbumin in yields up to 90 per cent and about 1 per cent of nitrogen was released in the trichloracetic acid soluble peptide form. The peptide was identified as Ala-Gly-Val-Asp-Ala-Ala (*3–5*).

According to Ottesen the transformation of ovalbumin into plakalbumin consisted of at least two steps, the first step being the opening of a single peptide bond of closed chain in ovalbumin molecule, leading to the formation of an open chain intermediate, and by the subsequent reaction the product is converted into plakalbumin, of which N-terminal is serine.

The author attempted to have more exact information about the nature of the peptide and protein formed by the action of bacterial proteinase upon native ovalbumin. It was found that the incubation of native ovalbumin with a bacterial proteinase (BPN′) (*6*, *7*) prepared from culture media of *Bacillus subtilis* N′ liberated three peptides and produced a new protein bearing threonine at N-terminal and proline (probably also valine) at C-terminal. The new protein crystallized not in plates but in rods. For the purpose of convenience the author named this protein H-albumin suggesting an open chain structure to this new protein. The present paper deals with the studies on the chemical nature of H-albumin and amino acid composition of the peptides liberated from ovalbumin by the action of BPN′.

EXPERIMENTAL

Materials. Ovalbumin—Ovalbumin was prepared by the method of Sørensen (*8*) and Cannan (*9*), crystallized five times, dialized against distilled water and lyophilized.

Bacterial Proteinase (*BPN′*)—This crystalline enzyme was obtained from *B. subtilis* N′

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and offered by Dr. Matsubara (Osaka University).

Digestion of Native Ovalbumin by Bacterial Proteinase (BPN′)—Five times crystallized ovalbumin (100 mg.) was dissolved in 10 ml. of distilled water (protein concentration: 1 per cent). The solution was brought to pH 6.3 with saturated barium hydroxide solution and to it was added 10 ml. of enzyme solution at 16°. The enzyme solution employed contained 0.1 mg. BPN′/ml. For quantitative estimation, 1 ml. of the reaction mixture was pipetted out and 1 ml. of 20 per cent trichloracetic acid was added. After 30 min., the solution was filtered and 0.5 ml. of the filterate was neutralized roughly with 0.32 ml. of *N* sodium hydroxide solution, then 1 ml. of 0.2 *M* citrate buffer (pH 5.0) was added and the ninhydrin reaction test was performed as described below. To each sample was added 0.1 ml. of stannous chloride solution (2 g. of $SnCl_2 \cdot 2H_2O$ in 124 ml. of 0.2 *M* citrate buffer of pH 5.0), 2 ml. of ninhydrin solution (1 g. ninhydrin in a mixture of 50 ml. of methyl cellosolve and 50 ml. of 0.2 *M* citrate buffer (pH 5.0)) and heated at 100° for 30 min. The solution was cooled rapidly, 5 ml. of diluent (ethanol: water; 1 : 1 v/v) was added and the optical density at 570 mμ was measured spectrophotometrically. The increase of the optical density of ninhydrin reaction of the soluble product was shown in Fig. 1. The digestion was performed for 3 hours in the subsequent preparative experiments.

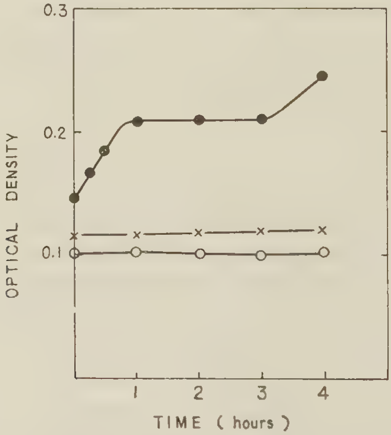


Fig. 1. Optical density of ninhydrin reaction of TCA-supernatant. —●—; Ovalbumin+BPN′; —×—, Ovalbumin only —○—, BPN′ only.

Isolation of New Protein from Native Ovalbumin—Five times crystallized ovalbumin (2 g.) was dissloved in 20 ml. of distilled water (protein concentration 10 per cent). The protein solution was brought to pH 6.3 with a saturated barium hydroxide solution, 2 mg. of BPN′ in 20 ml. of distilled water was added and the mixture placed at 20° for 3 hours. The reaction mixture was brought to pH 5 with 2 *N* hydrochloric acid and 15 g. of ammonium sulfate was added whereby the solution became turbid. When the protein solution was placed at room temperature for two or three days, rod crystals appeared slowly. These were repeatedly recrystallized by dissolving in water and with addition of ammonium sulfate. After five times crystallization, 500 mg. of apparently pure crystalline protein was obtained. It was freed of salt by dialysis for 5 days against distilled water and lyophilized for the following experiments.

Fractionation of Liberated Polypeptides—Digestion of native ovalbumin was carried out in the same way as described above, trichloracetic acid soluble part of the digest was separated by centrifugation, neutralized with sodium hydroxide and concentrated *in vacuo.* A part of the solution was analyzed by two dimensional paper chromatography. The solvents used were *n*-butanol-methylethylketone-water-17 *N* ammonia (5 : 3 : 1 : 1) for the first dimension and *n*-butanol-glacial acetic acid-water (4 : 1 : 1) for the second dimension. The remaining part of the peptide solution was dinitrophenylated by DNFB as usual and DNP-peptides produced were subjected to two dimensional paper chromatography using *n*-butanol saturated with 0.1 per cent ammonia for the first dimension and 1.5 *M* phosphate buffer (pH 6.0) for the second dimension. Chromatograms obtained are represented by Fig. 2a and b.

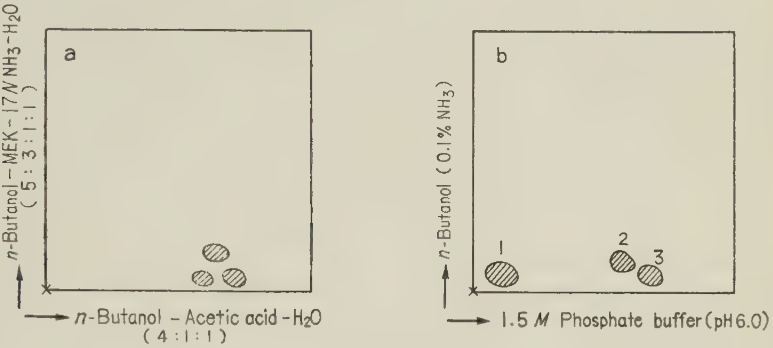


Fig. 2. Paper chromatograms of TCA soluble fraction. a—Chromatogram of peptides; colour developed by ninhydrin b—Chromatogram of DNP-peptides

Identification of N-Terminal Amino Acids of Peptides—For the identification of N-terminal amino acids of each peptide, DNP-peptides were fractionated by paper chromatography and three spots (1,2 and 3) on the paper were cut off and separately extracted by 1 per cent sodium bicarbonate solution and the extracts were evaporated *in vacuo* and hydrolyzed by heating with 5.8 *N* distilled hydrochloric acid for 17 hours at 105°. The hydrolyzates were diluted with equal volume of water and extracted three times with ethyl acetate. DNP-amino acids obtained were characterized by two dimensional chromatography as in the case of DNP-peptides described above.

Amino Acid Analysis of Liberated Peptides—The digestion of native ovalbumin by BPN′ was carried out as described above. To the digest was added equal volume of 20 per cent trichloroacetic acid. After one hour, the supernatant solution separated by centrifugation was neutralized with sodium hydroxide and evaporated *in vacuo.* The residue was hydrolyzed with 2 ml. of distilled hydrochloric acid (5.7 *N*) at 105° for 24 or 48 hours. The hydrolyzate was evaporated *in vacuo* to remove hydrochloric acid and used for amino acid analysis.

A small portion of hydrolyzate was dissolved in a few drops of water and the free amino acids were identified by two dimensional paper chromatography. The solvents used were *n*-butanol-methylethylketone-water-17 *N* ammonia (5 : 3 : 1 : 1 v/v) for the first dimension and *n*-butanol-glacial acetic acid-water (4 : 1 : 1 v/v) for the second dimension (*10*). Proline test by isatin reaction was found negative.

An aliquot of hydrolyzate was dissolved in 1 ml. of distilled water, neutralized with solid sodium bicarbonate, added with 2 ml. of 4 per cent sodium bicarbonate solution and 6 ml. of an ethanolic solution of DNFB (0.5 ml.). After shaking for 3 hours in a dark room, the reaction mixture was transferred to a separatory funnel with 20 ml. of water, extracted with 2×5 ml. of ether to remove excess DNFB, acidified with 2 N hydrochloric acid and extracted with 10, 5 and 3 ml. of ethylacetate. The organic and aqueous fractions were evaporated separately *in vacuo* and dinitrophenol was sublimed from the residue at 100° below 15 mm-Hg with a cold finger containing a piece of dry-ice. The nonvolatile residuals were then subjected to paper chromatography. The solvents used for organic fraction were *n*-butanol saturated with 0.1 per cent ammonia for the first dimension and 1.5 M phosphate buffer (pH 6.0) (1 M NaH_2PO_4+0.5 M Na_2HPO_4) for the second dimension. In the case of aqueous fraction, *n*-butanol-glacial acetic acid-water (4 : 1 : 1 v/v) which was used for the usual analysis of amino acids, was employed as the solvent of paper chromatography. For the quantitative estimation, each spot on paper was cut off, put in 5 ml. of 1 per cent sodium bicarbonate solution, heated at 55–60° for 15 min. and filtered. The optical density at 360 mμ of the filterate was estimated spectrophotometrically. In the case of spots near proline, the ultraviolet absorption spectrum was determined in order to make sure whether these spots were actually proline.

Identification of N-Terminal Amino Acids of the New Protein—The identification of N-terminal amino acids was carried out by Sanger's DNP-method (*11*). 100 mg. of protein were dissolved in 15 ml. of 4 per cent sodium bicarbonate and 0.2 ml. of dinitrofluorobenzene in 30 ml. of ethanol was added. The mixture was shaken for 3 hours at room temperature (25°), acidified with 2 N hydrochloric acid and centrifuged to precipitate the DNP-protein. The precipitate was washed exhaustively with water until the washings showed no Cl^- reaction. The precipitate was then washed three times with 30 ml. portions of 99 per cent alcohol, twice with 30 ml. portions of ether and dried *in vacuo* in a desiccator. Yield was about 100 mg.

Fifty milligrams of the DNP-protein were hydrolyzed with 2 ml. of distilled hydrochloric acid (5.8 N) at 105° for 8 or 17 hours. The solution was cooled to room temperature, 2 ml. of water was added and extracted with 3×4 ml. of ethylacetate. The combined extracts were evaporated and the residue was analyzed on DNP-paper chromatogram in the same way as described above. The aqueous layer was also evaporated to remove hydrochloric acid and chromatographed on paper. For the reconfirmation, each DNP-amino acid was converted into free amino acids by alkaline hydrolysis and identified by two dimensional paper chromatography.

Identification of C-Terminal Amino Acids—The identification of C-terminal amino acids was done by the hydrazinolysis-DNP-method reported by Akabori *et al.* (*12*) and by carboxypeptidase method.

a) Hydrazinolysis-DNP-method

To one hundred milligrams of protein was added 0.5 ml. anhydrous hydrazine and kept at 100° for 8 or 15 hours. The reaction mixture was evaporated *in vacuo* over sulfuric acid to remove excess hydrazine. The hydrazinolyzate was dissolved in 20 ml. of 4 per cent sodium bicarbonate solution and added with 0.5 ml. dinitrofluorobenzene in 40 ml. of ethanol. The mixture was shaken for 3 hours in a dark room at room temperature, diluted with 100 ml. of water and acidified with 2 N hydrochloric acid. The solution was then extracted with 50, 30 and 20 ml. of ethylacetate. The ethylacetate layer was extracted five times with 100 ml. portions of 2 per cent sodium bicarbonate solution in order to separate DNP-amino acids from DNP-amino acid hydrazides. The bicarbonate solution was washed with 100 ml. portions of ethylacetate and was acidified with 2 N hydrochloric acid. The solution was again extracted with 100, 50 and 30 ml. of ethyl acetate. The final ethylacetate extract was combined, evaporated *in vacuo* and from the residue dinitrophenol was removed by a cold finger.

The nonvolatile residue was dissolved in acetone and subjected to two dimensional paper chromatography. Eluates from each spot were determined of their optical density.

b) Carboxypeptidase method

Fourty five milligrams of protein were dissolved in 5 ml. of water and incubated at 37° with 0.02 ml. of a suspension (50 mg. per ml.) of commercial crystalline DFP-treated carboxypeptidase (Worthington Biochemical Corp.); the pH of the solution was adjusted to approximately 8.5 with 0.3 N potassium hydroxide. Aliquots of the reaction mixture were removed at intervals and shaked for 10 min. with addition of Dowex 50×8. After filtering, the exchanger was washed three times with water and then eluted with 3 ml. of 5 N ammonia. The eluted solution was evaporated *in vacuo* and the residue was subjected to paper chromatography.

Determination of Lysine in Ovalbumin and H-Albumin—The aqueous phase of the hydrolysates of DNP-protein, after being extracted with ethylacetate, was dried *in vacuo*. Five milliliters of methanol was added to the residue and sodium chloride was removed by filtration. The methanol solution was evaporated *in vacuo*, ε-DNP-lysine was separated by paper chromatography and determined quantitatively by electro-photometry. The solvent used was *n*-butanol-glacial acetic acid-water (4 : 1 : 1 v/v). The absence of mono-DNP-arginine and di-DNP-histidine in the aqueous phase was confirmed.

RESULTS AND DISCUSSION

On incubating native ovalbumin with a bacterial proteinase BPN′ prepared from culture media of *Bacillus subtilis* N′ a new protein was obtained, which is different from plakalbumin reported by Linderstrφm-Lang and Ottesen (*1*). It was found that no free amino acids but three peptides were released from ovalbumin by BPN′ digestion of ovalbumin in the trichloroacetic acid soluble fraction. The constituent amino acids in the whole peptides were determined after complete hydrolysis by two dimensional paper chromatography and the presence of Asp, Glu, Gly, Ser, Ala, Thr, Phe, Val, Leu or Ileu, but no proline was confirmed.

With regard to N-terminal amino acids of the liberated peptides serine was found in one of the three peptides and aspartic or glutamic acid in the other two peptides. Owing to the scanty yield of the material amino acid sequences of the peptides could not be determined.

Quantitative estimation of individual amino acids contained in the hydrolysate of whole peptides was carried out by two dimensional paper chromatography of dinitrophenylated hydrolysate according to the method of Levy (*13*) with a modification of solvents. Results of the analyses are shown in Table I. From figures in the table it could be assumed that 20 moles of amino acids were liberated in the form of peptides. The sum of molecular weights of these amino acid residues is approximately 2,000, and therefore, molecular weight of H-albumin must be ca. 43,000 assuming molecular weight of ovalbumin 45,000.

It was reported that native ovalbumin had no N-terminal amino acid (*14*, *15*) and this was confirmed by the present author. On the C-terminal, it was previously reported that alanine was found by thiohydantoin (*16*) and carboxypeptidase method (*17*). On the other hand, Steinberg (*18*) and Rocker (*19*) reported that native ovalbumin was not attacked by DFP-treated carboxypeptidase. They suggested that the initial attack of a DFP-sensitive, contaminated enzyme upon ovalbumin opened a single peptide bond in the closed chain of the ovalbumin molecule, giving rise to the C-terminal alanine. Niu and Fraenkel-Conrat (*20*) reported, however, that proline was found as C-terminal of ovalbumin by hydrazinolysis method. The same authors found the sequence Val-Ser-Pro for the C-terminal peptide. These results could also be confirmed by the present author (*21*).

TABLE I

Constituent Amino Acids of Peptides Liberated by BPN′ Digestion of Native Ovalbumin.

Exp.	I			II			III		
amino acid	optical density	molar ratio		optical density	molar ratio		optical density	molar ratio	
Glu	0.325	2.9	3	0.238	3.0	3	0.058	1.0	1
Asp							0.112	1.9	2
Gly	0.195	1.7	2	0.133	1.7	2	0.098	1.6	2
Ser	0.250	2.2	2	0.171	2.1	2	0.141	2.3	2
Ala	0.377	3.4	3	0.291	3.6	4	0.228	3.8	4
Thr	0.092	0.9	1	0.088	1.1	1	0.056	0.9	1
Phe	0.182	1.7	2	0.085	1.1	1	0.054	0.9	1
Val	0.612	5.8	6	0.442	5.5	6	0.207	3.4	3
Leu							0.198	3.3	3
Lys	0.208	1.3	1	0.112	1.0	1	0.080	0.9	1
Total			20			20			20

TABLE II

Free Amino Acids Resulted by the Hydrazinolysis of Ovalbumin and H-Albumin

Protein	Ovalbumin		New Protein	
Time of hydrazinolysis / amino acid	hrs. 8	15	8	15
Asp+Glu	0.1	0.3	0.0	0.1
Gly	0.2	0.2	0.2	0.2
Ser	0.2	0.2	0.2	0.3
Ala	0.2	0.3	0.1	0.2
Thr	0.1	0.2	0.1	0.1
Pro	0.6	0.8	0.6	0.6
Val	0.0	0.0	0.3	0.2

It is noteworthy that proline was not found in the hydrolysate of the peptide fraction, indicating that the peptides can not be derived from C-terminal of ovalbumin. Sugar moiety of ovalbumin could be detected only in H-albumin, but not in the trichloracetic acid soluble fraction of the digest.

C-Terminal determination of ovalbumin and H-albumin by hydrazinolysis according to Akabori *et al.* *(12)* are in good agreement with the discussion described above as shown in Table II.

Amino acids liberated from ovalbumin and H-albumin by the action of DFP-treated carboxypeptidase (Worthington Biochemical Corp.) are shown in Table III.

TABLE III

Amino Acids Liberated by the Action of DFP-Treated Carboxypeptidase

Incubation time hrs. / Protein	0	0.5	1.0
Ovalbumin	none	none	Gly, Ser, Ala
H-albumin	none	Val, Leu, Ala, Ser.	Val, Leu, Gly, Ala, Ser.

Results presented in Table II and III indicate the presence of proline, and probably also valine, at carboxyl terminal of H-albumin. The presence of one N-terminal amino acid, threonine, could be cleary demonstrated by Sanger's method as shown in Table IV.

TABLE IV

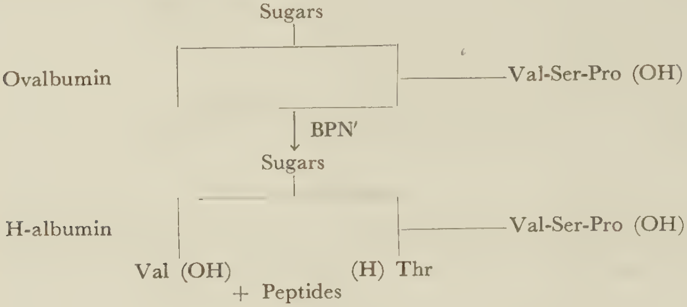
*Estimation of N-Terminal Threonine of H-Albumin*1)

Time of hydrolysis (hrs.)	DNP-Protein (mg.)	Protein2)		N-terminal threonine liberated		
		mg.	10^{-7} M	Found (10^{-7} M)	Threonine / Protein (uncorrected)	Threonine3) / Protein (corrected)
8	43.0	39	9.1	6.65	0.73	0.81
8	50.0	45	10.5	7.20	0.69	0.77
17	50.0	45	10.5	8.15	0.78	0.87
17	50.0	45	10.5	8.50	0.80	0.90

1) The molecular weight of new protein is assumed to be 43,000.
2) The content of protein of DNP-protein is assumed to be 90%.
3) Corrected by the factor of recovery of threonine after hydrolysis (0.90).

Lysine contents of ovalbumin and H-albumin are found to be almost the same *i.e.* 20 moles per mole protein. This is in good agreement with the fact that only one mole lysine among 20 moles of total lysine was liberated from ovalbumin by the action of BPN′.

From results and discussions described above it is highly probable that H-albumin bears one N-terminal threonine and two C-terminal amino acids, namely valine and proline, another N-terminal being masked in some unknown way. BPN′ digestion of native ovalbumin should, therefore, be expressed by the following scheme.



SUMMARY

1. A new protein, named H-albumin, was obtained in crystalline form from native ovalbumin incubated with a bacterial proteinase BPN′ accompanied with the liberation of three peptides in trichloracetic acid soluble form.

2. The composition of amino acids of the whole peptides is Glu$_1$, Asp$_2$, Gly$_2$, Ser$_2$, Ala$_4$, Thr$_1$, Phe$_1$, Val$_3$, (Leu or Ileu)$_3$, Lys$_1$.

3. The new protein was suggested to have one N-terminal (Thr) and two C-termineals (Val and Pro).

The author's thank is due to Dr. Jirgensons who kindly supplied with DFP-treated carboxypeptidase and to Prof. Akabori, by whose continuous encouragement the present work has been conducted.

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THE USE OF ERIOCHROME BLUE S.E.
(Mordant Blue 13; C.I. 16680)
FOR THE DETERMINATION OF CALCIUM IN SALIVA

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(Received for publication, November 18, 1959)

The classical methods employed for the determination of calcium in saliva and other body fluids are generally tedious and difficult. In 1955 a simple, rapid method for the analysis of calcium was reported by Yanagisawa (*1*). In this method a blue-purple dye, Eriochrome Blue S. E. (Mordant Blue 13; C.I. 16680; the disodium salt of 3-(5-chloro-2-hydroxyphenylazo)-4,5-dihydroxynaphthalene-2,7-disulfonic acid) was used as a metal sensitive indicator. Since then this indicator has been used for the determination of calcium in blood, urine, and feces by Flaschka (*2*), Kingsley (*3*) and Rich (*4*). The object of this study was to determine the suitability of this dye method for the estimation of calcium in saliva.

Aqueous solutions of Eriochrome Blue S.E. are magenta red in acid and bluish violet at and above pH 10. At this pH red-colored metal complexes form. The advantage of Eriochrome Blue S.E. over Eriochrome Black T and other eriochrome dyes is its relative stability at high alkalinities. In strong alkaline solutions Mg forms $Mg(OH)_2$, thereby making the reagent particles available to the calcium ions present in solution. The solution is titrated with ethylenediaminetetraacetic acid (EDTA). "The Metal-Indicator complex (Me·In) is weaker than the EDTA complex of the metal (Me) being titrated. During the titration, free metal ions are progressively complexed with EDTA. Finally, the metal is displaced from its indicator complex liberating the free indicator. (In)". (*2*)

$$\underset{\text{(blue)}}{\text{Me} + \text{Free Indicator}} \longrightarrow \underset{\text{(red)}}{\text{(Me·In Complex)}}$$

$$\underset{\text{(red)}}{\text{(Me·In Complex)}} + \text{EDTA} \longrightarrow \text{(Me·EDTA Complex)} + \underset{\text{(blue)}}{\text{Free Indicator}}$$

Since the end-point between the Me·In complex and the Free Indicator is difficult to see, the addition of the EDTA and the mixing of the titration solution is done directly in a Coleman Junior Spectrophotometer. (Fig. 1)

In a typical titration* 2.5 ml. of 2 *N* NaOH and 0.2 ml. of a 25 mg. per cent aqueous solution of Eriochrome Blue S.E. are added to 0.5 ml. of saliva and titrated at 610 mμ in a Coleman Junior Spectrophotometer with a solution containing 0.18 g./liter EDTA Blanks, and standards containing calcium in a concentration equivalent to 10 mg. per cent are routinely run. A sample titration curve follows. (Fig. 2)

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* The method reported herein is based on that of C. Rich, Rockefeller Institute for Medical Research. Since this method has not yet been published, it is summarized in this paper. A similar method was described, in abstract form, by Munson *et al.* (*5*).

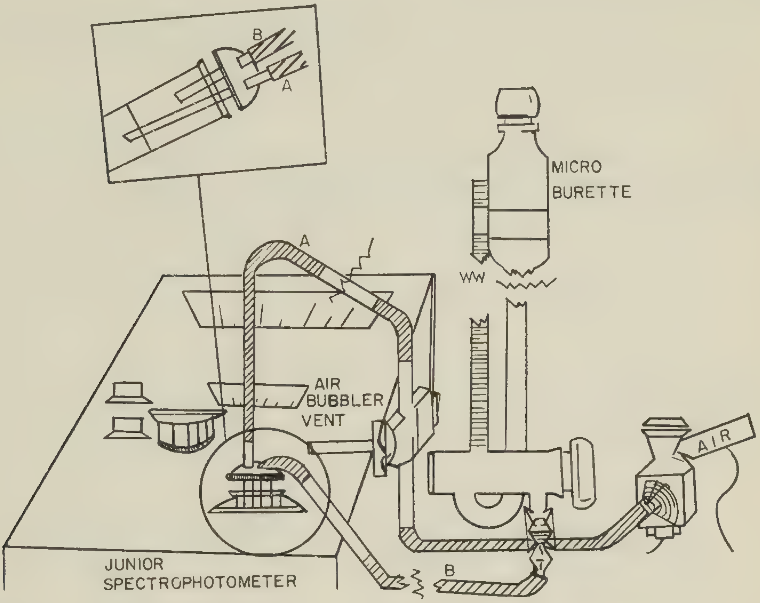

Fig. 1. Diagrammatic Sketch of the Apparatus Designed for Use in Titrating Directly into a Coleman Junior Spectrophotometer

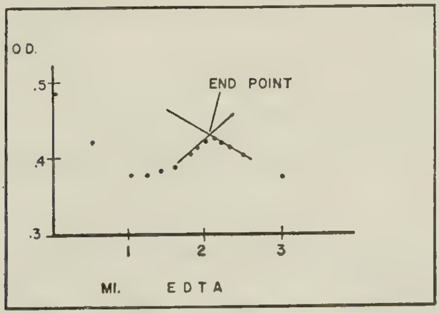

Fig. 2. Typical Titration Curve Obtained with the Eriochrome Blue S. E. Method

The intersect of two straight lines taken through the steepest part of the ascending and descending parts of the curve is the end-point. The net end-point for the standard and samples is obtained by subtracting the value of the blank titration. This curve is thought to represent a balance between the dilution of the metal-indicator complex, the appearance of the free indicator, and its subsequent dilution.

$$\text{mg.\% Ca of Sample} = \frac{\text{Net titration of sample} \times \text{mg.\% of Ca in Std. Sol.}}{\text{Net titration of Std.}}$$

RESULTS

1. Standard Curve—Calcium standards varying in concentration from 0.5 to 20 mg. per cent obeyed Beer's Law. (Fig. 3)

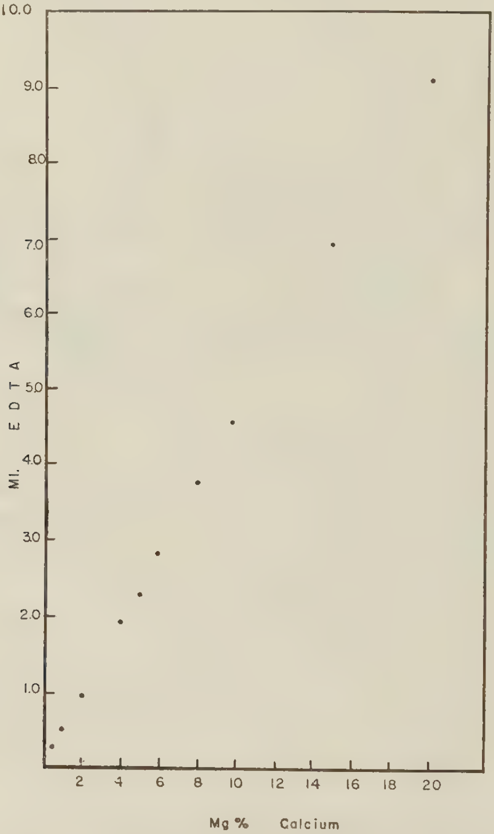

FIG. 3. Relation of Concentration of Calcium to ml. EDTA added

2. pH Effects—Alkaline standards made up in 0.5, 1.0, 2.0, 4.0 and 6.0 *N* NaOH did not affect the determination of calcium.

3. Color Stability—The Eriochrome Blue S.E. standard is stable up to one month at room temperature. The color formed after the addition of 2 *N* NaOH during the titration procedure is stable for approximately 5 to 10 minutes. In the procedure to be described, Eriochrome Blue is added at the time of the titration.

4. *Effect of Magnesium and Phosphorous*—$MgCl_2$ in final concentrations of 1–5 mg. per cent and P varying in final concentrations from 2–20 mg. per cent did not affect the titration for calcium.

5. *Mucin*—Gastric mucin in a final concentration of 0.3 per cent which approximates the concentrations of mucin found in whole saliva did not affect the determination for calcium.

6. *Recovery Determinations*—One to 10 mg. per cent of calcium were added to 0.5 ml. of pooled saliva and the specimens were analyzed by the Eriochrome Blue S.E.-EDTA procedure. Ninety-eight to 100 per cent of the amount added was recovered.

7. *Time*—The time required to determine calcium by the direct Eriochrome Blue S.E. method on whole saliva (see #8 below) is about 10∼12 minutes.

8. *Determinations on Saliva*—Calcium was determined on whole or digested* paraffin-stimulated saliva by a Direct and Indirect Eriochrome Blue S.E.-EDTA method and compared with results obtained by the Kramer-Tisdall method. (*6*)

A. Reagents—Make all reagents in conductivity water.

(1) Di-sodium, di-hydrogen EDTA ("Versene")—0.18 g./liter
(2) Eriochrome Blue S.E.** 25 mg. per cent
(3) 2 *N* NaOH
(4) Calcium standard solution, 10.0 mg. per cent
(5) 0.1 per cent methyl red in 95 per cent ethanol
(6) 5 per cent NH_4OH and 5 per cent HAc in dropper bottle (1 : 1 ratio)
(7) 3 per cent Ammonium Oxalate

B. Procedure in Eriochrome Blue S.E.-EDTA Titrations

(1) *Direct Method, Whole Saliva*—Place 0.5 ml. of well shaken saliva in a test tube. Add 2.5 ml. of 2 *N* NaOH and 0.2 ml. of the Eriochrome Blue S.E. solution. Titrate within 5 minutes at 610 mμ with EDTA in the Coleman Junior Spectrophotometer.

(2) *Direct Method, Digested Saliva*—Adjust 1.0 ml. of digested saliva to a salmon-pink color in the presence of methyl red with the NH_4OH-HAc mixture and prepare as above.

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* *Digestion Procedure*: Place 10 ml. of saliva, 2.5 ml. of concentrated H_2SO_4 and 10∼12 glass beads in a 50 ml. Erlenmeyer flask. Heat on a low flame until H_2SO_4 fumes appear and boiling ceases. Cool for 5 minutes, add 4 drops of 30 per cent H_2O_2 and heat until the digest is clear and colorless. Wash the digest into a 25 ml. volumetric flask and dilute with conductivity water. One ml. of the digest is equivalent to 0.4 ml. of saliva. Ten ml. of conductivity water and 10 ml. of the standard solution are treated in the same manner as the saliva sample.

** Eriochrome Blue S. E., Geigy Company, Basel, Switzerland; may also be obtained from Clinton Laboratories, 6010 Wilshire Blvd., Los Angeles 36, California (dye called Corinth Ca) and from the Inabata Industry and Company, Ltd., 2-chome Junkeimachi Minamiku, Osaka, Japan (dye called Plasmacorinth B).

(3) *Indirect Method, Whole Saliva*—Adjust 0.5 ml. of whole saliva and 3.5 ml. H_2O with 5 per cent NH_4OH using the methyl red indicator. Add 2 ml. of 3 per cent Ammonium oxalate, mix well and let stand overnight. Centrifuge and decant. Add 2 drops of concentrated HCl, 1.0 ml. of conductivity H_2O, 2.5 ml. 2 N NaOH and 0.2 ml. Eriochrome Blue S. E. Titrate with EDTA

(4) *Indirect Method, Digested Saliva*—Adjust 1.0 ml. of digested saliva and prepare as above.

0.5 ml. and 1 ml. blanks and standards are run on the whole and digested salivas respectively.

It is important to equalize the volumes of the blanks, standards and unknowns with conductivity water. In the direct method this should be done prior to the titration; in the indirect method, prior to the addition of the ammonium oxalate.

C. *Results on Saliva*

(1) *Variation among duplicate determinations*—Ten sets of duplicate determinations were made by the Eriochrome Blue S. E. and Kramer-Tisdall methods. The maximal variations between duplicates in the Kramer-Tisdall, the Direct Eriochrome Blue S. E., and the Indirect Eriochrome Blue S. E. methods on

TABLE I

Comparison of the Eriochrome Blue S. E. and Kramer-Tisdall Methods for the Determination of Salivary Calcium

	DIRECT ERIOCHROME BLUE S. E.		INDIRECT ERIOCHROME BLUE S. E.		KRAMER-TISDALL
	Whole Saliva	Digested Saliva	Whole Saliva	Digested Saliva	
	Calcium values expressed in mg.%				
1	4.26	4.22	6.40	4.42	4.46
2	4.08	4.04	4.80	4.24	4.26
3	5.60	5.72	6.60	5.76	5.68
4	5.55	5.49	5.70	5.54	5.27
5	4.08	4.00	4.60	4.06	4.26
6	5.47	5.25	6.00	5.00	5.47
7	5.55	5.48	6.80	5.56	5.67
8	3.52	4.00	6.60	3.75	4.06
9	4.08	4.10	5.60	4.06	4.25
10	6.11	6.18	8.20	6.25	6.10

digested saliva was 3.4 per cent. The variation seen in the Indirect Eriochrome Blue S. E. method on whole saliva was 8.2 per cent.

(2) *Salivary Calcium*—The results of the Eriochrome Blue S. E. and Kramer-Tisdall methods of the untreated or digested samples of stimulated whole saliva from the ten subjects are shown in Table I.

An examination of the values listed in this table suggests that, in general, the Direct Eriochrome Blue S. E. methods on untreated or digested whole saliva and the Indirect Eriochrome Blue S. E. method on digested whole saliva agree fairly well with the results obtained by the Kramer-Tisdall method. The Indirect Eriochrome Blue S. E. method on fresh whole saliva, however, appeared to give higher and more variable results.

To determine the validity of these impressions, a statistical evaluation of the data was performed.

The first test employed was an Analysis of Variance in order to determine if, indeed, there was a significant difference between the results of the various methods used. The results of this test indicated that the probability was less than 1 per cent that this magnitude of difference could have occurred by chance.

In view of these findings, a series of Paired "t" Tests was employed in order to relate the various Eriochrome Blue S. E. methods with the Kramer-Tisdall method, our standard of comparison. These tests showed that there is a significant difference between the Indirect Eriochrome Blue S. E. method on untreated whole saliva and the Kramer-Tisdall method, but no significant differences between the latter and the other Eriochrome Blue S. E. procedures.

In terms of accuracy, there was little choice between four of the five methods compared. The Direct Eriochrome Blue S. E. method on untreated whole saliva is, however, much simpler to perform and takes less time. (Table II.) The Direct Eriochrome Blue S. E. method is therefore the one of choice. No obvious explanation has been found for the consistently high values obtained for the calcium level by the Indirect Eriochrome Blue S. E. method on untreated whole saliva.

TABLE II

Comparison of the Approximate Time Required for Different Methods of Calcium Determination

(1)	Direct Eriochrome Blue S.E. Method-Untreated Whole Saliva	20 min.
(2)	Direct Eriochrome Blue S.E. Method-Digested Whole Saliva	2 hrs.
(3)	Indirect Eriochrome Blue S.E. Method-Untreated Whole Saliva	2½ hrs.[1]
(4)	Indirect Eriochrome Blue S.E. Method-Digested Whole Saliva	3½ hrs.[1]
(5)	Kramer-Tisdall Method-Digested Whole Saliva	4 hrs.[1]

1) 1 hour allowed for calcium oxalate precipitation. Overnight is preferable.

CONCLUSIONS

A simple, rapid, accurate, direct titrimetric dye method on whole saliva—employing Eriochrome Blue S. E. (Mordant Blue 13; C. I. 16680)—has been adapted for the determination of calcium in saliva. Reasonable concentrations of magnesium, phosphate, and mucin do not interfere; the color is fairly stable; and the results agree well with the Kramer-Tisdall method.

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The Journal of Biochemistry, Vol. 47, No. 6, 1960

A METHOD FOR THE SEPARATION OF TRICARBOXYLIC CYCLE INTERMEDIATES BY CELITE-COLUMN CHROMATOGRAPHY AND ITS APPLICATION TO THE TISSUES OF ASCARIS LUMBRICOIDES VAR. SUIS*

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(Received for publication, November 20, 1959)

Methods for the isolation and the determination of tricarboxylic acid-cycle intermediates from a single sample of tissue by means of column chromatography are not yet fully established, although some success has been obtained by using silica gel (*2*), ion exchange resin (*3*), or Celite (*4*).

Prior to the last paper (*4*), the Celite column was successfully used in separating organic acids from plant or ascaris, by Bulen *et al.* (*5*) and Bueding and Yale (*6*).

Being suggested by these works (*5, 6*), an attempt was made in the present paper to apply Celite to the separation and the measurement of tricarboxylic acid-cycle members in tissues. The results obtained by this method made it possible to analyze the members of tricarboxylic acid-cycle in the tissues of *Ascaris lumbricoides* var. *suis*, in which there is still considerable doubt as to the presence of the tricarboxylic acid-cycle.

METHODS

The procedure for column preparation was essentially the same as that used by Bulen *et al.* (*5*). Five grams of Celite (Johns-Manville, No. 535), washed with ether and dried at 100° for 5 hours or more, was mixed with 2 ml. of 0.2 N H_2SO_4, stirred throughly with a glass rod, suspended in 30 ml. of dry chloroform, and then packed into a chromatographic tube (10 mm. × 30 cm.), the suspension of Celite was poured in to the column in 4 or 5 portions, the surface being slightly pressed with a glass rod after each application to make the final 5-6 cm. length of the zone. (see also DISCUSSION)

One milliliter of sample to be tested, acidified with a few drops of 5 N H_2SO_4, was mixed with about 1 g. of Celite, added to about 5 ml. of chloroform; then, this mixture was put on the top of the column***.

Various organic solvents were examined as eluting agents: ethanol, *n*-butanol, isoamyl alcohol, ethyl acetate, benzene, *n*-hexane, chloroform and acetone. Of these agents isoamyl alcohol–chloroform and *n*-butanol—chloroform mixtures were found to be suitable for the present purpose*. They consisted of chloroform solution containing 1, 5, 10, 20, 25, 30, or 35 per cent (V/V) of *n*-butanol and 10 per cent (V/V) of isoamyl alcohol. Fifty milliliters of each mixed solvent were put on the top of the column successively in the order of above-mentioned ratios, and the effluent was collected at the rate of 5 ml. per 3 minutes. Each fraction of 5 ml. was titrated with 0.002 *N* KOH solution, after adding 2 ml. of carbonate-free water and 2 drops of 0.005 per cent methanol solution of Cresol Red. CO_2-free air was passed through the mixture throughout the titration.

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* Presented in part at the 18th Meeting of the Japanese Pharmacological Society (Tokyo, July 22, 1958) (*1*).

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*** Chloroform effluent contains fatty acids, their esters, and sterol derivatives.

RESULTS

The standard sequence of the acids in effluent was determined by chromatographing the authentic specimens of the acids. A typical chromatogram of the standard sequence is presented in Fig. 1.

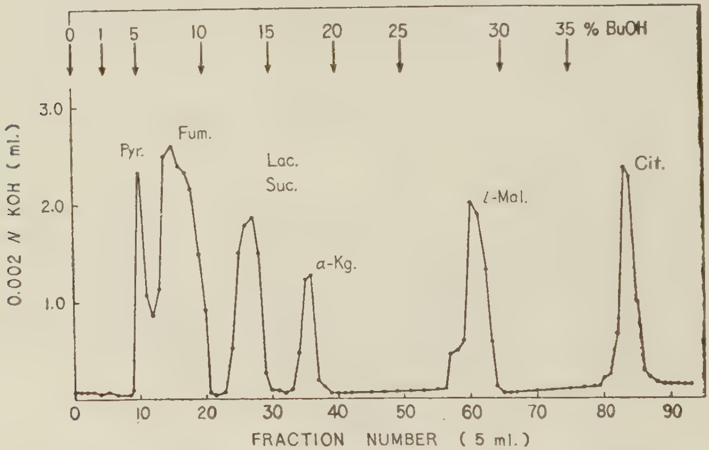

Fig. 1. A typical chromatogram for separation of mixture of authentic acids (chloroform-butanol system).

All the organic acids in tricarboxylic acid cycle were separated from one another except succinic acid and lactic acid. The separation of succinic acid and lactic acid was performed by rechromatographing the aqueous layer of the fractions containing both acids, after concentrating its volume to 1 ml., through a second column using isoamyl alcohol-chloroform mixture as the elution solvent (Fig. 2).

The quantitative aspect of this experiment is summarized in Table I. Identification of each acid was made as follows: lactic acid by Barker-Summerson's method (*7*), succinic acid by succinoxidase system of pigeon breast muscle (*8*), α-keto acids by paper chromatography as 2,4-dinitrophenyl hydrazones (developed by a mixture consisting of butanol: ethanol: 10 per cent $NH_4OH = 7:1:2$ (*9*)), L-malic and other di- and tri-carboxylic acids by paper chromatography as fluorescent compounds (Table II).

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* Ethyl acetate was decomposed during development, giving a high blank value; ethanol dehydrated the column; benzene, acetone and *n*-hexane increased tailing.

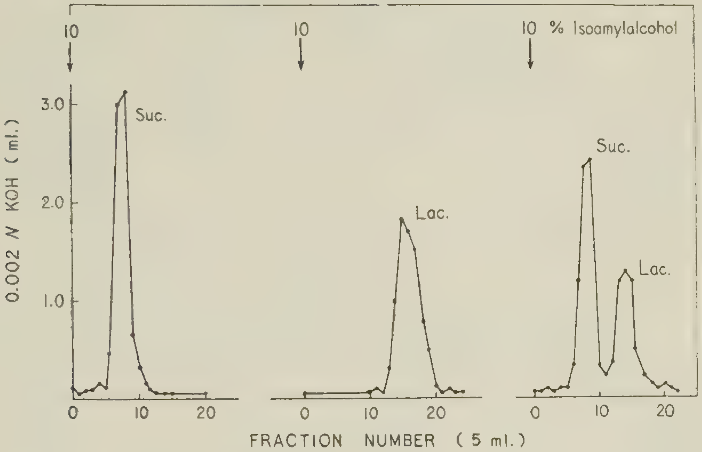

FIG. 2. A chromatogram for separation of succinic acid and lactic acid (chloroform-isoamyl alcohol system).

TABLE I

Recovery of Organic Acids from Known Mixtures

Acid	No. of Exp.	Chloroform-*n*-Butanol	Chloroform-iso-Amylalc.
		%	%
Formic	2	92.0	
Pyruvic	3	82.0	
Fumaric	3	83.2	
Succinic	3	92.1	90.1
Lactic	3		84.2
α-Ketoglutaric	2	85.0	
L-Malic	4	94.3	
cis-Aconitic	3	81.0	
Citric	3	87.6	

Then the chromatographic method described above was applied to rat tissues. The results summerized in Table III are in good accord with the previous reports by other workers (*10*, *11*).

With the backgrounds mentioned above the perienteric fluid and muscle of ascaris was analyzed by this method. The results are shown in Table IV.

TABLE II

Paper Chromatography of Organic Acids as Fluorescent Compounds

Acid		R_f EtOH 7 H_2O 3	R_f n-BuOH 4 AcOH 1 H_2O 2	Color of fluorescence
L-Malic	β-Naphthol	0.71		Violet
Fumaric	Resorcinol		0.66	Blue violet
Succinic			0.56	Light blue
L-Malic			0.36	Sky blue
α-Ketoglutaric			0.35	Light blue
Oxalacetic				Blue green
cis-Aconitic				Light blue
Citric			0.49	Sky blue

TABLE III

Contents of Organic Acids in Tissues of Normal Rat

Acid	Brain	Kidney	Liver
Pyruvic	17.7	18	50
Fumaric	122	35	53
Succinic	60	9	10
Lactic	796	229	118
Oxalacetic	45	7	8
α-Ketoglutaric	134	15	16
L-Malic	16	9	13
cis-Aconitic	0.2	nil	nil
Citric	22	11	15

μM/100 g

TABLE IV

Contents of Organic Acids in Ascaris

Acid	Perientric Fluid	Muscle
Pyruvic	0.09	0.68
Fumaric	nil	nil
Succinic	3.17	21.50
Lactic	0.32	0.23
Oxalacetic	0.14	nil
α-Ketoglutaric	nil	1.20
L-Malic	nil	nil
cis-Aconitic	nil	nil
Citric	nil	nil

μM/100 g

DISCUSSION

In our experience*, silicic acid column chromatography was not suitable for separating the organic acid of Krebs cycle because of its poor reproducibility. In this respect the Celite column was shown to be the superior to the silicic acid column, because it would give constant results irrespective of the number of the lot of the Celite preparation, if the ratio of 0.2 *N* H_2SO_4 to Celite, the rate of flow, and the length of column were all made constant.

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* unpublished data

Bulen *et al.* (*5*) Buch *et al.* (*3*) and Swin *et al.* (*4*) equilibrated each solvent mixture against dilute sulfuric acid solution before use. According to the present author's experience*, this treatment was found to be the chief factor causing some variability in the elution pattern.

The results of this paper show that most of each organic acid involved in tricarboxylic acid-cycle were separated from one another with a recovery in the range of 80 to 90 per cent. It must be noted, however, that oxalacetic acid showed a very broad peak in 5 per cent *n*-butanol-chloroform fraction, so it was difficult to determine the exact amount of pyruvic acid in the presence of oxalacetic acid. Acetoacetic acid was completely destroyed during packing and developing.

Recently, Bueding and Farrow (*12*) identified succinic acid as the component of acidic fraction in perienteric fluid of ascaris. This was confirmed by this paper. Furthermore, as shown in Table IV, a large amount of this acid was also found in muscle tissue. This may suggest some defects in succinoxidase system or the presence of powerful assimilatory system of succinic acid other than Krebs cycle, but at present the origin and the nature of the succinic acid are in still unknown. Oya (*13*), using well-washed muscle homogenates, founds a potential oxidation of succinic acid and its acceleration by methylene blue: the inhibiting effect of malonic acid on the succinate oxidation was observed, although it depressed the endogenous oxidation. He has supposed that succinic acid metabolism in ascaris is quite different from other animal tissues.

No significant tricarboxylic acids could be found, but it does not necessarily mean that the tricarboxylic acid-cycle is not operative in ascaris tissues, because Oya (*13*) has observed the increase of O_2 uptake when the well-washed homogenate of ascaris muscle was incubated with α-ketoglutaric acid or a low concentration of citric acid. Furthermore, citric acid was synthesized from oxalacetic acid and acetyl-CoA by particulate fraction of muscles**.

Very low concentrations of fumaric and L-malic acids would be explained in part by the abundant presence of malic enzyme, (*13*, *14*). But a more sensitive method to determine the amount of these acids is needed for further investigations.

SUMMARY

1. A method of column chromatography was described, in which 0.2 *N* H_2SO_4 on Celite was used as the stationary phase and *n*-butanol-chloroform or isoamyl alcohol-chloroform mixtures as the solvent system.

2. Main intermediates of the tricarboxylic acid-cycle were separated by

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* unpublished data

** unpublished date (Ueno, 1959)

the above mentioned two solvent systems and determined by titration with 0.002 *N* KOH. The recovery of the acids ranged from 80 to 90 per cent.

3. This method was applied to the determination of the amount of members of the tricarboxylic acid-cycle in tissues of normal rat. The results were in good accord with the previous reports obtained by different procedures.

4. Application of the above mentioned method to the tissues of *Ascaris lumbricoides* var. *suis* showed that succinic acid was the main component in muscle as well as perienteric fluid, whereas no significant amount of tricarboxylic acids were discovered in these tissues. Problems concerning metabolism in ascaris were discussed from this unique feature in distribution of the acids found.

The authors wish to express their thanks to Prof. Y. Kobayashi and to Dr. S. Ebashi for their continued interest in this work, and to Dr. T. Tatsuno for synthesizing α-ketoglutaric and oxalacetic acids. The authors were also indebted to Miss K. Hamano for her technical assistance.

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STUDIES ON LIPOPROTEIN LIPASE

I. DETERMINATION OF LIPOPROTEIN LIPASE

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(Received for publication, November 21, 1959)

Many reports have been published on the method of determining lipoprotein lipase (also called the lipemia clearing factor). In 1955 Korn (*1*) reported a method for determining this enzyme by estimating the glycerol which was liberated during the enzymatic reaction.

The present work was undertaken to determine the activity of lipoprotein lipase in plasma. The estimation was made by a modified method of the colorimetry of glycerol after the deproteinization of the enzymatic reaction mixture.

MATERIALS AND METHODS

Heparin—Sodium salt of heparin (100,000 units per 89.3 mg.) (Daiichi Kagaku Co., Ltd.) was used throughout the experiments.

Enzyme Solution—Five ml. of blood was drawn into a centrifuge tube containing 0.5 ml. of 3.2 per cent sodium citrate solution after an intravenous injection of heparin had been given to fasting animals, and plasma was obtained, after being centrifuged at 5,000 r.p.m. for 10 minutes at 4°. In the case of the rat, blood was obtained by cardiac puncture under the light anesthesia with ether, and from the dog by venepuncture.

Substrate—Olive oil (J. P. VI) was emulsified to 15 per cent (w/v) by 0.5 per cent aqueous solution of Tween 60 and diluted to 3 per cent (w/v) with distilled water before use.

Incubation Mixture—A mixture of 1 ml. of enzyme solution, 1 ml. of 3 per cent oil emulsion, 2.5 ml. of 0.1 N ammonia-ammonium chloride buffer (pH 8.0) containing $M/25$ calcium chloride, and 0.5 ml. of 5 per cent aqueous solution of bovine serum albumin (Armour's Fraction V) was incubated at 37°. One ml. of incubation mixture was pipetted out at the beginning and then at regular intervals throughout the incubation period and mixed with 2 ml. of 10 per cent trichloroacetic acid. After standing for 30 minutes at room temperature, precipitates were filtered off and the filtrate was used for determination of glycerol.

Colorimetric Determination of Glycerol—Determination of glycerol was done by a modified method of Lambert and Neish (*2*) described below. A half ml. of the filtrate was pipetted into a calibrated, stoppered test tube and oxidized by addition of 4 ml. of 0.025 M KIO_4 solution. After 5 minutes 0.1 ml. of 0.5 M sodium arsenite was added and the remaining excess periodate was reduced. After standing for 10 minutes 9 ml. of chromotropic acid reagent were added and the mixture was thoroughly shaken and heated for 30 minutes in a boiling water bath. After cooling, the coloured solution was filled up to 11 ml. with water and determined at 570 mμ in a photoelectric colorimeter using a 10 mm. cuvette. The oxidation of glycerol by periodate was as successful in trichloroacetic acid solution as in the sulphuric acid solution used by the above authors.

RESULTS AND DISCUSSION

As shown in Fig. 1, a known amount of glycerol gave different extinction values according to the content of plasma protein by a modified Lambert and Neish method using sulphuric acid, as indicated by Korn (*1*). However, the present trichloroacetic acid method gave the same extinction values of pure glycerol solution, irrespective of the plasma protein content as shown in Fig. 2. From these data, the extinction value of 0.100 corresponded to 2.1 μmoles of pure glycerol in 5 ml. of the incubation mixture.

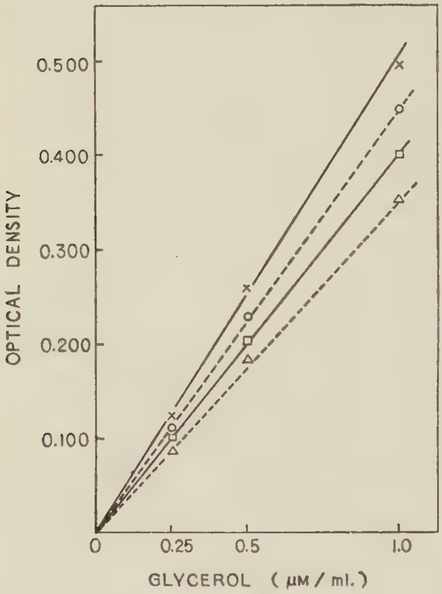

Fig. 1. Disturbance of plasma protein for recovery of glycerol. One ml. of standard solution of glycerol containing various proportions of dog plasma pipetted into 2 ml. of N-H_2SO_4. A half ml. of acidified mixture was taken for estimation of glycerol by the method described by Korn (*1*) (—×—) 0.5 ml. of H_2O. (—○—) 0.1 ml. of plasma + 0.4 ml. of H_2O, (—□—) 0.2 ml. of plasma + 0.3 ml. of H_2O, (—△—) 0.4 ml. of plasma + 0.1 ml. of H_2O, were added to 0.5 ml. of standard solution of glycerol, respectively.

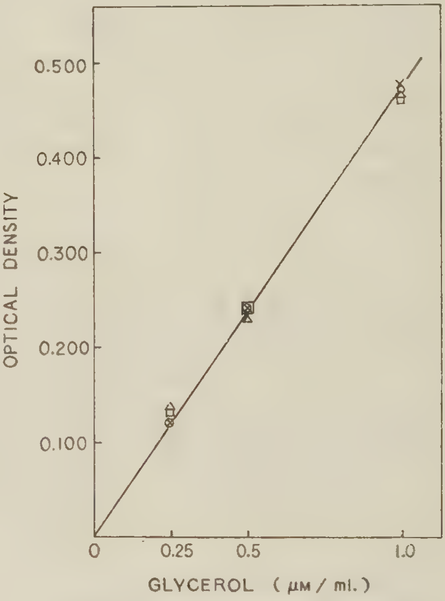

Fig. 2. Determination of glycerol in plasma after deproteinization with trichloroacetic acid. One ml. of standard solution of glycerol containing various proportions of dog plasma poured into 2 ml. of 10% trichloroacetic acid. After removing the precipitates, 0.5 ml. of filtrate was taken for assaying glycerol by the authors' method. The same plasma-containing standard glycerol solution being used, the symbols were the same as shown in Fig. 1.

The amount of liberated glycerol was linear to both the incubation time (within two hours) and the amount of enzyme solution as shown in Fig. 3. For convenience two hours incubation period was employed in most of the subsequent experiments.

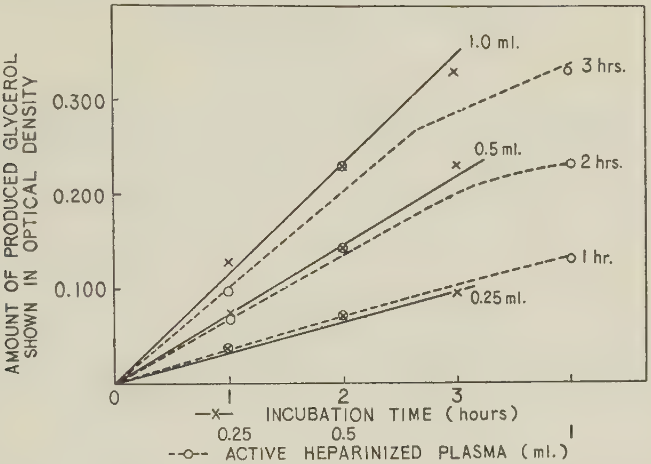

Fig. 3. Amounts of produced glycerol were linear to both the incubation time (within 2 hrs.) and the amount of enzyme solution. One ml. of enzyme solution (containing 0.25 ml., 0.5 ml. and 1 ml. of active heparinized dog plasma, respectively) was added to 4 ml. of incubation mixture, and 1 ml. of the mixture was pipetted off after incubation of 0, 1, 2, and 3 hrs. and lipoprotein lipase activities were determined by estimation of glycerol by the authors' method.

Table I

Inhibition of Lipoprotein Lipase in the Presence of Higher Concentrations of Salts, and Protamine Sulphate

Amounts of inhibitors in final concentration		Lipoprotein lipase activity Δ O. D. at 570 mμ
NaCl	0	0.043
	0.348 *M*	0.060
	0.696 *M*	0
$CaCl_2$	0	0.050
	0.02 *M*	0.135
	0.2 *M*	0
Protomine sulphate	0	0.124
	26.2 μg./ml.	0.089
	140.52 μg./ml.	0.086
	260.1 μg./ml.	0.065

As enzymatic activity was not apparent in the preheparin serum obtained under normal experimental conditions, and furthermore, as shown in Table

I, the enzymatic activity of heparinized plasma was inhibited in higher ionic strength or by the addition of protamine sulphate as previously reported by other investigators (*1*, *3*), the present method described by the authors was suitable for the determination of the lipoprotein lipase which was liberated in blood plasma after injection or secretion of heparin.

SUMMARY

A modified method for the quantitative assay of lipoprotein lipase based on the determination of glycerol has been presented. After the deproteinization of enzymatic reaction mixture with trichloroacetic acid, the filtrate obtained was oxidized with periodate and the resulting formaldehyde was coloured with chromotropic acid without disturbance.

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THE BIOSYNTHESIS OF VITAMIN B_{12} AND PORPHYRINS BY PROPIONIBACTERIUM

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The hypothesis on the identity of the mechanism of vitamin B_{12} and porphyrins biosynthesis suggested by Shemin (*1*) on the ground of a substantial similarity in the structure of the molecules of these substances is an extremely inviting one. This line of reasoning is supported by the inclusions into the molecule of both compounds of such substances as acetate and glycine (*2*), δ-aminolevulinic acid (ALA) (*1*, *3*) and of porphobilinogen (*4*).

However, besides the features similar to porphyrins as regards the structure vitamin B_{12} posesses a series of peculiarities of its own (*1*), which suggests us the possibility of a difference in the ways of the biosynthesis.

The present work was undertaken with the purpose of clearing up this problem. The study was concerned with the culture of *Propionibacterium shermanii*, which is interesting since alongside the vitamin B_{12} it forms a large amount of free porphyrins (*5*), mainly coproporphyrin.

In a way of a working hypothesis we have assumed that the initial stages of porphyrin and B_{12} biosynthesis are identical and therefore we are equally right in classing the ALA as a precursor of vitamin B_{12} as of the porphyrins.

To verify this supposition, we have synthesized the ALA after a method suggested by Shemin and have tested its action upon the culture of *Propionibacterium.*

It was found that the addition of 5–20 mg./100 ml. of ALA to a peptone nutritive medium caused a considerable increase (3∼6-fold) in the synthesized porphyrins and did not effect upon the amount of vitamin, that is, the organism quite easily turns the ALA into porphyrins, and does not use it for building up of the vitamin molecule.

Having recognized this we have decided to alter the process so as to stimulate the predominating formation of vitamin at the expense of porphyrins hindering the formation of the latter.

Cobalt and ferrous salts have been chosen for this purpose since it is known that cobalt enters into a molecule of the vitamin B_{12} and stimulates its formation, while ferrous salt is a strong depressant of porphyrin biosynthesis (*6*).

It has been observed that in the absence of cobalt the organism accumulates similarly great amounts of porphyrins and does not accumulate any B_{12}. However, the addition of minimum amounts of cobalt (1 mg. $CoCl_2 \cdot 6H_2O$/100 ml.) is sufficient to ensure the normal formation of vitamin, whereas any further increase in the dosage of salts of cobalt does not entail an increase in the vitamin yield, affecting merely the quality of the newly formed porphyrins, namely when 10 mg. of cobalt chloride was added to 100 ml. there occurred a marked change in the spectral characteristic of the porphyrins obtained: the absorption peak shifted from 401–405 mμ to 415 mμ (Fig. 1).

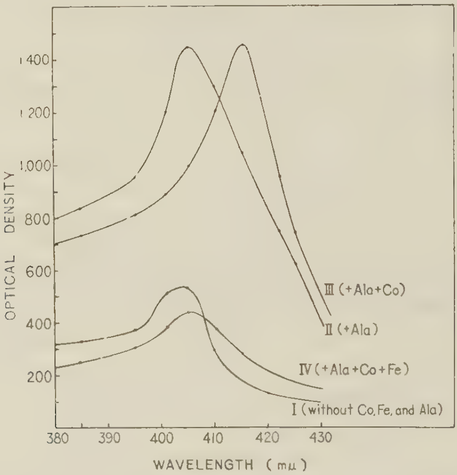

Fig. 1. Absorption spectra of the porphyrins. The influence of additions to porphyrin formation; B_{12} in I and II-0, III-6.8, IV-6.7 mg./liter. See the text.

In all probablility, cobalt as a strong complexing agent, under these conditions, enters a porphyrin molecule forming metallo-porphyrin. The ease of this sort of reactions is attested to by the data of McConnel (7) who has obtained various artificial Co-porphyrins in vitro by ordinary porphyrin treatment by salts of cobalt.

The incorporation of cobalt in porphyrin has brought about the complete loss of one of the characteristic properties of free porphyrins—the capability of being extracted by ether from an acidified aqueous solution (in our case from a cultural medium). The presence of cobalt in metallo-porphyrins has been confirmed by a qualitative test with nitroso-R-salt. All these data permit us to believe that in the presence of increased doses of cobalt, the culture of *Propionibacterium*, indeed synthesizes Co-porphyrin in large amounts.

As to the ferrous salt an amount of 400 μg. per 100 ml. in the form of $FeSO_4 \cdot 7H_2O$ suffices to reduce noticeably the formation of porphyrin, even when such an effective porphyrin precursor as the ALA was added to the medium. The further increase in ferrous salt brought about an even greater inhibition of porphyrin biosynthesis although it was not accompanied by the expected increase in vitamin B_{12} (Table I).

TABLE I

The Effect of Fe^{++} upon B_{12} and Porphyrin Biosynthesis

Addition to 100 ml. of medium.	weight of cells g./liter	B_{12} mg./liter	porphyrins mg./liter
Control (I)	4.3	7.8	9.5
I+10 mg. ALA (II)	4.2	7.6	45.0
II+1 mg. $FeSO_4$ $7H_2O$	4.3	7.5	40.0
II+2 mg. ,, ,,	4.1	7.3	33.0
II+5 mg. ,, ,,	4.2	7.0	20.0
II+10 mg. ,, ,,	4.3	6.0	15.0
II+25 mg. ,, ,,	4.1	5.5	3.5

Thus, the ALA is the structural material perfectly suitable for building porphyrins while it is not used for the formation of B_{12} under the indicated conditions.

A plausible explanation may be found in the idea offered by Shemin (*1*) namely that the B_{12} biosynthesis proceeds not from the ALA proper, but from its methylated derivate, inasmuch as the molecule of the vitamin is exceedingly rich in methyl groups.

Taking it into consideration we deemed it interesting to look into the methylation processes in relation to the biosynthesis of vitamin B_{12}.

It is generally recognized at present that the methylation in living organisms is effected by enzyme systems including active forms of the folic acid and the *p*-aminobenzoic acid (PAB), since it is a component part of a complex molecule of the folic acid.

TABLE II

Sulfathiazol Action upon B_{12} and Porphyrin Biosynthesis

Addition to 100 ml. of medium	B_{12} mg./liter	porphyrins mg./liter
I Initial medium	7.6	17.5
I+sulfathiasol 1 mg. (II)	5.7	13.2
II+folic acid 200 γ	5.5	13.0
II+folinic acid 200 γ	5.6	13.1
II+PAB 2 mg.	7.6	18.5
II+methionine 2 mg.	7.7	13.0

One of the most widespread procedures of studying the action of folic and PAB acids is the inhibition of their action by different antagonists and the removal of the inhibition by one or another metabolite.

As a PAB antagonist, we have used sulfathiazol, and aminopterin as an antagonist for folic acid. The effect of these substances has been traced against the formation of porphyrins and B_{12}. The antagonists themselves were introduced to the initial medium. Table II presents data for the sulfamide inhibition.

It turned out that sulfathiazol at a concentration of 1 mg. per cent causes noticeable depression of the formation of vitamin and porphyrins (by 20–25 per cent) without influence on the bacterial growth. Further increase in sulfathiazol brings about the inhibition of growth.

The inhibiting action of sulfathiazol is completely removed by PAB. The formation of B_{12} and porphyrins is restored to normal. Besides, the following possible methyl group donors were tested: methionine, threonine, serine, choline, betaine, sarcosine. Of all these substances only methionine had eliminated the inhibiting action of the sulfathiozol upon the formation of B_{12}. As to the porphyrin formation, the methionine does not eliminate the inhibiting influence of sulfathiazol upon the process.

To prove the supposed mechanism of sulfamide action upon the B_{12} biosynthesis through its influence upon the methylation processes a study was undertaken on the action of aminopterin which is a specific antagonist of folic acid (see Table III).

TABLE III

Aminopterin Action upon B_{12} and Porphyrin Biosynthesis

Addition to 100 ml. of medium	B_{12} mg./liter	porphyrins mg./liter
I Initial medium	8.3	16.0
I+aminopterin 4 mg. (II)	6.3	16.0
II+folic acid 200 γ	7.0	15.0
II+folinic acid 200 γ	8.2	15.5
II+methionine 2 mg	8.4	15.0

At a concentration of 5 mg. per cent aminopterin reduced the B_{12} yield by 20-25 per cent without affecting the formation of porphyrins and the accumulation of the bacterial cells. As should have been expected PAB did not remove this action, folic and particularly folinic acids were more effective while of the above stated donors of the methyl groups only the methionine proved to be effective. Thus, these findings demonstrate also the importance of methylation for the normal formation of vitamin B_{12}.

In connection with the results obtained it is necessary to mention the work of Shemin and collaborators (*8*) who have shown that carbon-labeled methyl group is incorporated into a B_{12} molecule only from methionine but not from betaine or choline. The authors believe that it is precisely this methyl group that goes to make the 6 "extra" methyl groups in a B_{12} molecule.

Our data stand in complete agreement with these results and give us the right to say that one of the possible and probable differences in the biosynthesis of B_{12} and porphyrins is precisely the process of methylation which has a very important part in the construction of the B_{12} molecule.

Therefore, it is possible to consider that one of the functions of the folic acid in an organism is its participation in the biosynthesis of B_{12}. So far it is hard to tell at which stage it begins to take part in the construction of a B_{12} molecule, whether it is by the methylation of the initial ALA or by transmitting the methyl group to the newly formed porphyrin structure, thus completing its building up to a B_{12}.

Another evidence of the difference in the biosynthesis of porphyrins and vitamin B_{12} is different sensitivity of these two processes to X-ray irradiation.

The irradiation in a dose of 30 kr. has been applied to a two-day old culture which was subsequently used as inoculum. An increase in the dose to 60 kr. has brought about the complete inhibition of the development of the culture grown on this culture medium. The results obtained are shown in Table IV.

TABLE IV

The Irradiation Effect (30 kr.) upon B_{12} and Porphyrin Biosynthesis

Addition to 100 ml. of medium	Weight of cells g./liter	B_{12} mg./liter	porphyrins mg./liter
Non irradiated inoculate (I)	4.25	7.1	15.2
Irradiated inoculate (II)	4.30	4.2	16.1
II+10 mg. ALA	4.20	4.0	35.3
II+methionine 10 mg.	4.30	6.2	15.4
II+folinic acid 300 γ	4.30	5.3	15.3

First of all the attention is drawn to the (40 per cent) considerable suppression of the B_{12} biosynthesis by a irradiated culture.

It is remarkable that as distinct from all this, the irradiation does not affect the porphyrin formation. A practically complete (88 per cent) normalization of B_{12} formation is caused by methionine, folinic acid has a weaker effect. The added ALA, in the irradiated culture too is used only for the formation of porphyrins increasing their yield by more than 2-fold, but it does not stimulate the vitamin accumulation at the same time.

The latter circumstance is interesting also in the following respect. The failure of the attempts at employing of ALA to increase the B_{12} yield could be explained by the existence of a peculiar physiological "top limit" for a cell, *i.e.* the presence of a maximum vitamin content which cannot be surpassed by the cell.

The irradiation data refuse this interpretation since under the conditions of an inhibited B_{12} biosynthesis too, the ALA is not used just the same for the construction of the additional amounts of the vitamin. Most likely the irradiation affects above all the methylation processes without influencing the ALA condensation reactions which bring to the construction of a ring-like porphyrin structure.

The studies which have been mentioned here improve our knowledge of the ways of vitamin B_{12} biosynthesis, corroborate the above mentioned hypothesis advanced by Shemin and set forth the task of obtaining and direct testing of the vitamin precursor of methylated aminolevulinic acid.

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AMINO ACID DECARBOXYLASES OF PROTEUS MORGANII

III. STIMULATORY ACTION OF GLUCOSE ON INDUCED FORMATION OF HISTIDINE DECARBOXYLASE*

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Some characteristics of the induced formation of amino acid decarboxylases of *Proteus morganii*, such as histidine, ornithine, and glutamic acid decarboxylases, have been reported in the previous papers (*1*, *2*) and it was also described that these amino acid decarboxylases were formed inducibly when the microorganism was incubated in the induction medium with glucose and ammonium salt as carbon and nitrogen sources in the presence of inducer. Further studies on the induced formation of histidine decarboxylase have revealed that, when *Pr. morganii* was incubated with amino acid mixture in place of glucose and ammonium salt in the presence of the inducer, the enzyme is formed inducibly, and that in this amino acids medium glucose, ribose, gluconate, and pyruvate stimulate the induced formation of the enzyme. Present paper deals with the stimulatory action of these compounds.

EXPERIMENTAL

Proteus morganii was grown in the synthetic basal medium as described previously (*1*). Cells were harvested at the post-exponential phase of growth (12 to 15 hours' incubation), and induced with 10^{-2} *M* of histidine as an inducer unless otherwise stated in the induction medium containing carbon and nitrogen sources. The cellular density was approximately 0.1 mg. dry weight of cells per ml.

Estimation of histidine decarboxylase activity was carried out as described previously (*1*) and the specific activity was expressed in terms of μl. CO_2 evolved per hour per mg. dry weight of cells. The rate of the formation of the enzyme was calculated as the increment of specific activity of the enzyme in the induction period, during which optical density of bacterial suspension increased by 0.10 scale. Bacterial cell growth was measured turbidimetrically with Coleman spectrophotometer at 600 mμ.

Casamino acid, being free from vitamins, was purchased from Difco Lavoratories Inc., Detroit, Michigan. Potassium gluconate was kindly supplied by Dr. H. Murooka of the Institute of Applied Microbiology, University of Tokyo.

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RESULTS

Bacterial Cell Growth and Formation of Histidine Decarboxylase—Washed cells of *Pr. morganii* grown in the synthetic basal medium were incubated in the induction medium containing 10^{-2} *M* histidine along with glucose and ammonium salt as carbon and nitrogen sources. As shown in Fig. 1, specific activity of histidine decarboxylase increased markedly and optical density of the suspension also increased almost linearly during incubation, and it was revealed that the increase in activity of the enzyme was in proportion to the increase in the optical density. When *Pr. morganii* was incubated in which fructose, galactose, ribose, gluconate, or pyruvate was added in place of glucose in the above mentioned induction medium, the rates of growth in respective media were found to be lower than that with glucose, and the optical density of bacterial suspensions increased linearly after a short lag phase in cases, as galactose, ribose, gluconate, or pyruvate was added (Fig. 1-a). However, the increase in histidine decarboxylase activity against the increase in the optical density (approximately, the increase in cell material) was the same rate as that with glucose, as shown in Fig. 1-b.

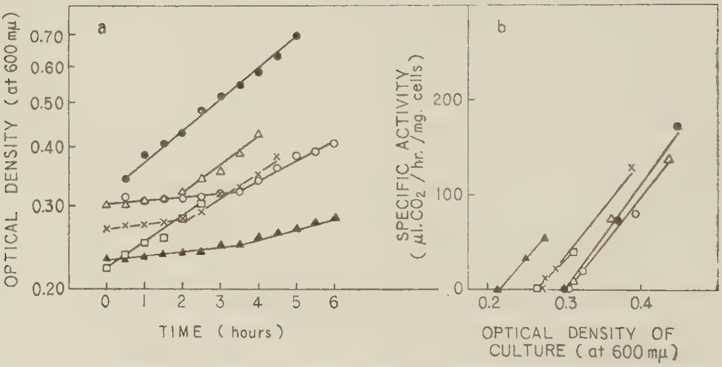

FIG. 1. Change in optical density and formation of histidine decarboxylase in *Proteus morganii*.

Induction medium contained 10^{-2} *M* carbon sources, such as glucose (—●—), gluconate (—△—), ribose (—×—), pyruvate (—○—), fructose (—□—), and galactose (—▲—), respectively.

Formation of Histidine Decarboxylase in Amino Acids Medium—In this experiment, *Pr. morganii* was incubated in the medium, to which were added amino acids as carbon and nitrogen sources in place of glucose and ammonium salt. Casamino acid was added for amino acid mixture in varied concentrations. Rates of cellular growth with 0.05 and 0.1 per cent casamino acid were observed to be 50 and 60 per cent of those with 0.5 and 1.0 per cent casamino acid, respectively. The results are shown in Fig. 2. It was observed that in amino acids medium the rate of enzyme formation increased with the increase in the concentration of casamino acid and attained maximum at a concentration of 1.0 per cent, while in induction media containing glucose, pyruvate or others as carbon sources the enzyme was formed at the same rate regardless to the rates of growth (Fig. 1).

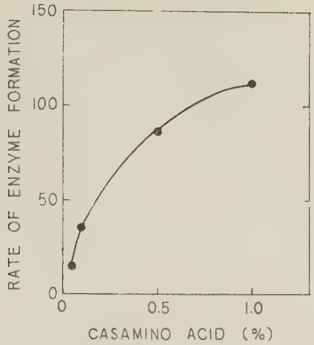

FIG. 2. Rate of formation of histidine decarboxylase in amino acids medium in *Proteus morganii*.

Induction medium contained 10^{-2} *M* histidine as an inducer and casamino acid for carbon and nitrogen sources, respectively. See table I for the rate of enzyme formation.

When washed cells were incubated in amino acids induction media which were supplemented with certain carbon compounds, it was found that the enzyme induction was markedly enhanced. The results indicated that the induced formation of histidine decarboxylase in the presence of these carbon compounds, such as glucose, pyruvate, ribose, or gluconate, was activated as much as 180–220 per cent of the control (Table I). The stimulations in the rate of growth of *Pr. morganii* were found to be fairly marked with glucose,

TABLE I

Rate of Formation of Histidine Decarboxylase in Amino Acids Medium in Proteus morganii

	Carbon compounds added				
	None	Glucose	Pyruvate	Ribose	Gluconate
Rate of enzyme formation	30	62	66	57	62
Relative rate of growth	100	260	100	120	160

Induction was performed in induction media containing 0.1 per cent casamino acid and 10^{-2} *M* histidine in the presence or in the absence of 5×10^{-3} *M* carbon compounds, respectively. The rate of enzyme formation was calculated as the increment of specific activity of the enzyme in the induction period, during which optical density of bacterial suspension increased by 0.10 scale. Relative rates of growth are expressed as percentages of the rate in the control.

slight with ribose and gluconate, and none with pyruvate. Therefore, it is probable to consider that glucose, pyruvate or others supply a factor activating the induction of the enzyme through metabolic reactions of these compounds.

Effect of Pyridoxal on Formation of Histidine Decarboxylase—Guirard and Snell established that pyridoxal phosphate has been found to participate in the form of coenzyme in histidine decarboxylase activity in the case of *Lactobacillus* (*3*). Histidine decarboxylase of *Pr. morganii* also requires this vitamin as coenzyme*. It is, therefore, considered that one of factors required for induction of the enzyme is the biosynthesis of the coenzyme. In attempt to determine whether this would be the case, cells were incubated in 0.1 per cent casamino acid medium with 10^{-3} M pyridoxal. The result was negative and no stimulation of the enzyme formation was marked, as shown in Table II.

TABLE II

Effect of Pyridoxal and ATP on Formation of Histidine Decarboxylase in Proteus morganii

	Compounds added		
	None	Pyridoxal	ATP
Rate of enzyme formation	32	30	32

Induction was performed in induction media containing 0.1% casamino acid and 10^{-2} M histidine in the presence or in the absence of 10^{-3} M pyridoxal or ATP, respectively. See table I for the rate of enzyme formation.

Effect of ATP on Formation of Histidine Decarboxylase—To the 0.1 per cent casamino acid medium was added 10^{-3} M adenosine triphosphate (ATP) and the cells were incubated in it. As shown in Table II, however, induction of the enzyme was found to be the same rate as the control.

Stimulating Activity of Glucose on Formation of Histidine Decarboxylase—To clarify the stimulatory action of glucose, pyruvate or others on the induced formation of histidine decarboxylase, rates of enzyme formation were measured in the presence of varied concentrations of glucose. Cells were incubated with 10^{-2} M histidine in media in which casamino acid was added at concentrations of 0.1, 0.5 and 1.0 per cent, respectively, and to each casamino acid medium were added varied concentrations of glucose. In the induction medium with 1.0 per cent casamino acid, no stimulation was observed and the rates in the presence of varied concentrations of glucose were found approximately to be the same value as the maximal one shown in Fig. 3. In Fig. 3, rates of the induction in 0.1 and 0.5 per cent casamino acid media are shown. It was revealed that maximal values observed in both media were the same, but the type of curves differed from each other, and the concentrations of glucose for 50 per cent maximal rate of the formation of the enzyme were found to be 4×10^{-4} *M* in 0.1 per cent and 1.2×10^{-4} *M* in 0.5 per cent of casamino acid, respectively. The results suggest that the biosynthesis of the enzyme, in each case, in the induction medium with amino acids was actively induced under the supply of more energy source.

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* Ando, S., unpublished experiments.

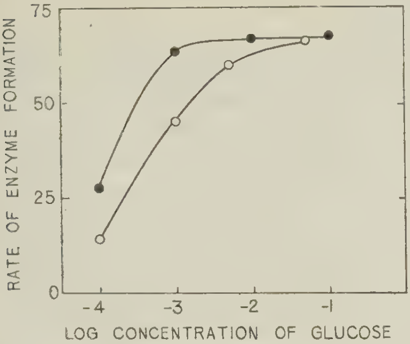

FIG. 3. Effect of glucose on formation of histidine decarboxylase in *Proteus morganii.*

Induction medium contained casemino acid, 10^{-2} *M* histidine, and varied concentrations of glucose. Casamino acid was added at concentrations of 0.1% (—○—) and 0.5% (—●—), respectively. See table I for the rate of enzyme formation.

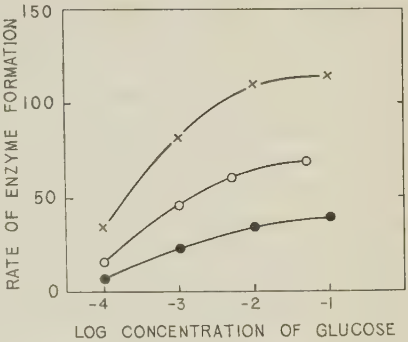

FIG. 4. Effect of glucose on formation of histidine decarboxylase in *Proteus morganii.*

Induction medium contained histidine, 0.1% casamino acid, and varied concentrations of glucose. Histidine was added at concentrations of 10^{-3} *M* (—●—), 10^{-2} *M* (—○—), and 10^{-1} *M* (—×—), respectively. See table I for the rate of enzyme formation.

To make more perceivable the relationship between energy supply and stimulatory action on the formation of the enzyme, the following experiment was carried out. Cells were incubated in 0.1 per cent casamino acid media containing varied concentrations of histidine and formation rates of the enzyme were estimated in respective casamino acid media in the presence of varied concentrations of glucose. As shown in Fig. 4, each curves were found to be similar type and the concentration of glucose for 50 per cent maximal rate of the formation of the enzyme fall in the same value in these cases, while maximal rates increased in proportion to the increase in the concentration of histidine.

DISCUSSION

Experimental results indicate that, although the growth is a necessary factor for the induction of histidine decarboxylase in *Pr. morganii*, the rate of the enzyme formation is not proportional to the rate of the growth. And also the result (Fig. 3) showed the requirement of satisfactory supply of energy source for the active induction of the enzyme.

There is a possible explanation of the mechanism with which the energy source when added stimulates the induction of histidine decarboxylase. As has been investigated by Gale with *Staphylococcus*, glutamic acid enters the cells only in the presence of glucose as energy source (*4*). If this would be the case with histidine, the concentration of glucose, at which the maximal rate of the formation of the enzyme was observed in casamino acid media containing varied concentrations of inducer, might be expected to increase in proportion to the increased concentration of the inducer. The result (Fig. 4) seems not to favor the possible mechanism described above. Moreover, in the present experiment, the growth of cells, which indicated the utilization of amino acids as energy sources added in the medium, was observed during the induction period. On the other hand, in the experiments by Gale, washed cells were suspended in salt buffer solution containing no sources of carbon and nitrogen and, therefore, cellular growth did not develop.

It is probable to consider alternatively that, although amino acids in the induction medium may be utilized sufficiently for the growth of cells, they are unable to provide an energy source satisfactorily for the induced formation of histidine decarboxylase. There are much works on the stimulation of enzyme formation by the addition of glucose (*5*, *6*, *7*) and in the presence of energy sources (*8*, *9*, *10*). Indeed, in *Pr. morganii* the stimulation of the enzyme induction was also marked by the addition of pyruvate, a possible metabolic intermediate of glucose, which was likely utilized for a substrate of energy forming system. Although it was found that the presence of ATP stimulated the formation of certain enzymes (*9*, *10*), no stimulation was observed in *Pr. morganii*. The discrepancy between these observations may be explained by the consideration that the entry of ATP into cells of *Pr. morganii* may be unable to carry out freely against the cell barrier.

The consideration stated above leads to the suggestion that for the induced biosynthesis of histidine decarboxylase a greater supply of energy source than that might be required for the formation of constitutive components is indispensable.

SUMMARY

When washed cells of *Proteus morganii* was incubated in induction media containing glucose, fructose, ribose, galactose, gluconate, or pyruvate, respectively, for carbon source, induced formation of histidine decarboxylase was performed at the same rate in respective media, while the rates of the growth of cells in induction media differed from each other.

Washed cells also formed inducibly the enzyme in an induction medium containing casamino acid for carbon and nitrogen sources. By the addition of certain compounds, such as glucose, ribose, gluconate, or pyruvate, to casamino acid medium the induction of the enzyme was stimulated markedly. The possible mechanism of the stimulatory action of these compounds were discussed.

The author wishes to thank Prof. S. Akabori and Prof. K. Miyaki for their interest and encouragement, and Prof. T. Sasakawa and Dr. M. Hayashi for their valuable advice and discussion.

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TURNIP PEROXIDASE

II. THE REACTION MECHANISMS OF TURNIP PEROXIDASES A_1, A_2 AND D

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In the preceding paper of this series (*1*), the existence of three kinds of peroxidases in turnip roots was demonstrated, and these peroxidases, designated as "TP-A_1**, TP-A_2** and TP-D**" were highly purified and studied as to their physicochemical properties. In this paper, the results of the kinetic studies on these enzymes are described and the mechanism of the reaction is discussed in comparison with that of HRP** established by Chance and George (*cf.* (*2*, *3*)).

MATERIALS AND METHODS

Materials—TP-A_1, TP-A_2 and TP-D used in the present study were prepared as described previously (*1*). These preparations were separately dissolved in a 0.1 M phosphate buffer (pH 7.0) to give a concentration of about 10^{-5} M, the concentrations of the solutions having been determined spectrophotometrically on the basis of the extinction coefficients given in the preceding paper, and stored in the cold. These stock solutions were diluted with water to about 10^{-6} to 10^{-8} M just before use, and 0.1 ml. of each of the resulting solutions was immediately used as a component of a reaction mixture as described below, since the activity of such an extremely dilute solution of peroxidase was found to decrease rather rapidly. Guaiacol and ascorbic acid were used as hydrogen donor. Guaiacol was purified by redistillation under reduced pressure. Ascorbic acid was a commercial crystalline sample purchased from the Wako Junyaku-kogyo Co. The extinction coefficients of ascorbic acid at 268 and 290 mμ were estimated by the present author to be 11.7 and 1.94 cm.$^{-1}$ mM^{-1} at pH 4.7, respectively, using 10 cm.$^{-1}$ mM^{-1} for the extinction coefficient at 268 mμ at pH 4.6, which was reported by Chance (*4*). Hydrogen peroxide used was of analytical grade obtained from the Edogawa Kagaku-kogyo Co. The water used was twice distilled with glass container.

Determination of the Rate of Reaction—Spectral measurements were made with a Beckman model DU spectrophotometer, equipped with a water-jacketted cell compartment maintained at 20°. The over-all reaction was followed by measuring the change in absorbance at 470 mμ when guaiacol is used for hydrogen donor and at 268 or 290 mμ when the hydrogen donor is ascorbic acid. The reaction was initiated by the addition of 0.027 ml. of aqueous hydrogen peroxide into the mixture of 3.1 ml. that contained 1.0 ml. of 0.1 *M* buffer solution 0.1 ml. of enzyme solution and varying amounts of hydrogen donor, and the absorbance was recorded at about 10 second intervals. The hydrogen peroxide was added as a drop at the end of stirring rod, with which the reaction mixture was stirred vigorously. The rate of over-all reaction is expressed by the following equation:

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** TP-A_1, TP-A_2 and TP-D stand for turnip peroxidases A_1, A_2 and D, respectively. HRP stands for horseradish peroxidase II.

$$v=-\frac{dx}{dt}=-\frac{da}{dt}\begin{cases}=\frac{1}{\varepsilon}\times\frac{dD}{dt} & \text{(for guaiacol)}\\ =-\frac{1}{\varepsilon}\times\frac{dD}{dt} & \text{(for ascorbic acid)}\end{cases}\qquad \text{Eq. (1)}$$

where x and a are the concentration of hydrogen peroxide and that of hydrogen donor in the reaction mixture, respectively, D the absorbance, and ε the molar extinction coefficient of guaiacol in the oxidized form* or that of ascorbic acid itself at the wavelength used. The initial rate of reaction, v_0, was calculated using the value of the tangent of the reaction-time curve at zero time. The experimental error which comes from this treatment was minimized by choosing an appropriate concentration of the enzyme to give the absorbance change of about 0.05 to 0.10 for 30 seconds, so that the curves might show as possible as small curvature.

RESULTS

Guaiacol as Hydrogen Donor—The initial rate of reaction catalyzed by TP-D was obtained at pH 7.0 and 20°, in various concentrations of hydrogen peroxide and guaiacol, the results being summarized in Fig. 1, where e/v_0 is plotted against $1/a_0$ at each x_0 (e, a_0 and x_0 standing for the initial concentrations of the enzyme, hydrogen donor, and hydrogen peroxide, respectively). As seen in this figure, these values fall in straight lines which are almost parallel to each other. When the intercepts of these lines on the ordinate ($e/v'_{\max}$)** are plotted against $1/x_0$, a straight line is again obtained as shown in Fig. 2. This line does not seem to pass the origin, even if the experimental errors are taken into consideration. Based on the results obtained, the rate of reaction may be expressed by the following equation:

$$\frac{e}{v_0}=c_1+c_2\times\frac{1}{x_0}+c_3\times\frac{1}{a_0}\qquad \text{Eq. (2)}$$

where c_1, c_2 and c_3 are coefficients which are independent of x_0 and a_0. The values of c_1, c_2 and c_3 of TP-D computed from the data illustrated in Figs. 1 and 2, are given in Table I.

Also in the case of TP-A_2, the relationships between the rate of reaction,

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* The molar extinction coefficient of guaiacol in the oxidized form was found by the present author to be 5.57 cm.$^{-1}$ mM^{-1} at pH 7.0, by means of a method similar to that of George (*5*), being slightly smaller than the value calculated from the data of his experiment at pH 5.4 (?), *i.e.* 6.67 cm.$^{-1}$ mM.$^{-1}$ This is the extinction coefficient per one millimole of guaiacol in the oxidized form per litre. Therefore, the rate of over-all reaction, v, can be written as Eq. (1), if only hydrogen peroxide oxidizes after all equimole of guaiacol, even though the substance produced from guaiacol were really tetraguaiacol (*6*) or dihydroxy-dimethoxy-diphenyl (*7*).

** "$v'_{\max}$" means the maximum rate at the infinite value of a_0 at fixed x_0.

x_0 and a_0 are represented by Eq. (2) as shown in Fig. 3. However, the lines obtained by plotting e/v_0 against $1/a_0$ are placed so close to each other that the values of the coefficients except c_3 have greater experimental errors than those of TP-D. A similar relation was also observed with in the case of TP-A_1. The values of the coefficients of these peroxidases are also presented in Table I.

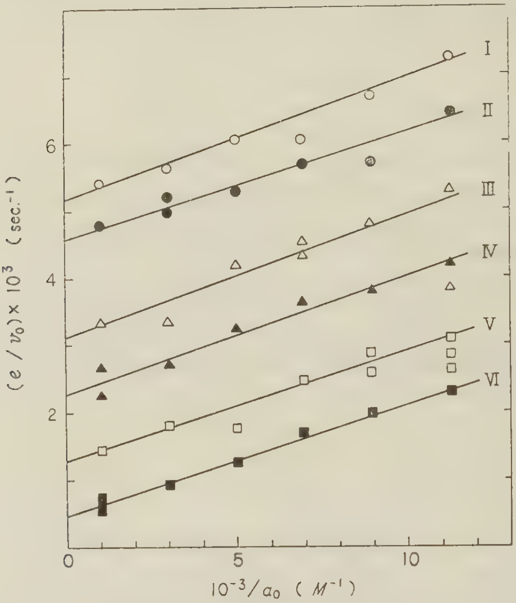

FIG. 1. Relation between e/v_0 and $1/a_0$ at fixed x_0. (turnip peroxidase D).

Initial rate of over-all reaction (v_0) catalyzed by turnip peroxidase D was measured, according to the method described in the text, using various concentrations of guaiacol at a series of fixed concentration of hydrogen peroxide (I-VI). Reciprocal of the rate per mole per litre of the enzyme, e/v_0, is plotted against $1/a_0$ for each experiment at the fixed hydrogen peroxide concentration. The concentration of hydrogen peroxide in the reaction mixture, x_0, was as follows: I, —○—, $3.66\times10^{-5}\,M$; II, —●—, $4.62\times10^{-5}\,M$; III, —△—, $6.63\times10^{-5}\,M$; IV, —▲—, $8.77\times10^{-5}\,M$; V, —□—, $2.10\times10^{-4}\,M$; VI, —■—, $1.06\times10^{-3}\,M$. The range of guaiacol concentration used in these experiments was $9.1\times10^{-5}\,M$ to $1.0\times10^{-3}\,M$. The range of concentration of enzyme in the reaction mixture, e, was $1.6\times10^{-10}\,M$ to $1.6\times10^{-9}\,M$. The measurements were made at pH 7.0 (phosphate buffer) and 20°.

Ascorbic Acid as Hydrogen Donor—Since the autoxidation of ascorbic acid could not be neglected in this experiment (pH 4.7), the rate of reaction was corrected by subtracting blank rate ascribed to the spontaneous oxidation of ascorbic acid. The observed values of v_0 did not show a satisfactory fit.

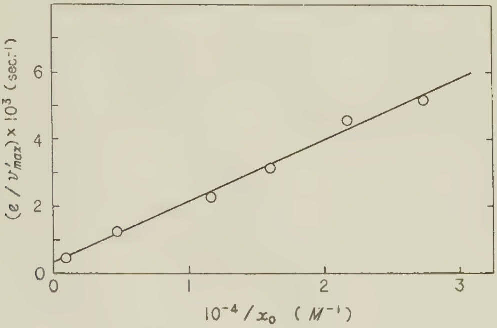

FIG. 2. The plots of $e/v'_{\max}$ versus $1/x_0$.
The values of $e/v'_{\max}$ were obtained from the intercepts of the each line in Fig. 1 to the ordinate.

However, when the values of e/v_0 were plotted against $1/a_0$ at fixed x_0, approximately straight lines were also obtained. The values of $e/v'_{\max}$ and the slope of those lines obtained by the least square method are presented in Table II. The plotting of $e/v'_{\max}$ against $1/x_0$ was omitted, since it was difficult to obtain accurate values corresponding to c_1 and c_2 of Eq. (2) because of considerable experimental error and close proximity of the line to each other.

TABLE I

The Values of the Coefficients of Eq. (2) and of the Rate Constants Obtained by the Use of Guaiacol as Hydrogen Donor

The values of c_1, c_2 and c_3 were obtained from the data of Figs. 1 and 2, and on the basis of these values, the values of the rate constants in this table were calculated in the manner described in the text. pH 7.0, 20°.

Coefficients and rate constants	TP-A_1	TP-A_2	TP-D	HRP[1]
c_1 (sec.)	1.5×10^{-3}	$1.2 \quad 10^{-3}$	3.2×10^{-4}	
c_2 (sec. M)	0.7×10^{-7}	5.5×10^{-7}	1.86×10^{-7}	
c_3 (sec. M)	2.2×10^{-5}	1.5×10^{-5}	1.69×10^{-7}	
k_1 (M^{-1}sec.$^{-1}$)	1×10^7	2×10^6	5.4×10^6	9.0×10^6
k_4 (M^{-1}sec.$^{-1}$)[2]	4.5×10^4	$6.8 \quad 10^4$	5.9×10^6	2.2×10^5(pH6.7)
k_9 (sec.$^{-1}$)[3]	0.7×10^3	0.8×10^3	3.1×10^3	
K_M (M^{-1})[3]	1.4×10^{-2}	1.3×10^{-2}	5.3×10^{-4}	

1) ref. (*10*)
2) assuming Mechanism A
3) assuming Mechanism B

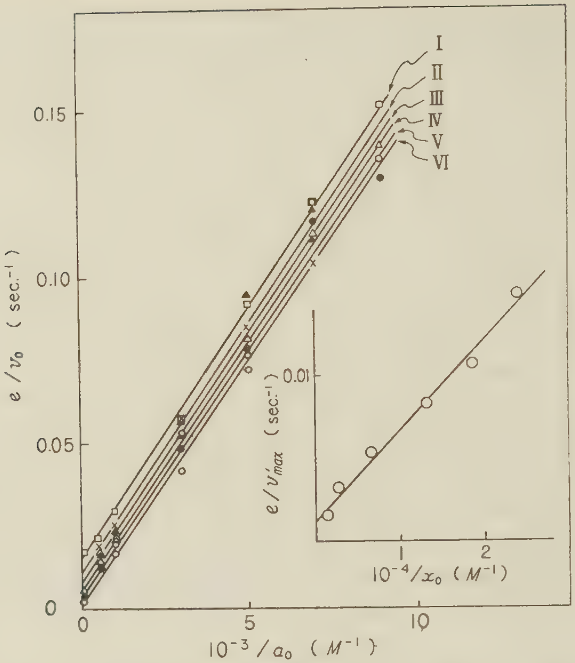

FIG. 3. The plots of e/v_0 versus $1/a_0$ at fixed x_0, and the plots of $e/v'_{\max}$ versus $1/x_0$. (turnip peroxidase A_2).

The method of the measurement of over-all reaction and that of the plotting of the observed values are essentially the same as that of Figs. 1 and 2. The concentration of hydrogen peroxide of each line was as follows: I, —□—, 4.24×10^{-5} *M*; II, —×—, 5.45×10^{-5} *M*; III, —▲—, 7.64×10^{-5} *M*; IV, —△—, 1.53×10^{-4} *M*; V, —●—, 3.82×10^{-4} *M*; VI, —○—, 7.64×10^{-4} *M*. The range of concentration of guaiacol used in these experiments was 1.1×10^{-4} *M* to 1.1×10^{-2} *M* and that of the enzyme was 3.0×10^{-9} *M* to 5×10^{-8} *M*. Experiments were carried out at pH 7.0, 20°.

DISCUSSION

As described in other papers of this series (*1*, *8*), it has been found in the case of the turnip peroxidases here used that the Complex I and II having absorption spectra similar to those of HRP are formed, and that the same reaction sequence as that of HRP, *i.e.* peroxidase → Complex I → Complex II → peroxidase, occurs. Accordingly, the mechanism of the reaction of the enzymes here used appears to be essentially of the same nature as that of HRP.

According to Chance *et al.* (*2*, *3*), the mechanism of the reaction catalyzed by HRP is represented in principle as follows:

Table II

Determination of.the Rate Constant k_4 for Asoorbic Acid

The initial rate of over-all reaction (v_0) catalyzed by TP-A_1, TP-A_2 and TP-D was measured, by the method described in the text, using various concentrations of ascorbic acid at the fixed concentration of hydrogen peroxide as shown in the second colomn of this table. The range of ascorbic acid concentration used was 1.8 to 15 mM for TP-A_1, 1.5 to 14 mM for TP-A_2, and 1.5 to 95 mM for TP-D. The reaction was carried out at 20° and pH 4.7 (acetate buffer of final concentration 32 mM). The values of $e/v'_{\max}$ and slope presented in this table were obtained by plotting the values of e/v_0 against $1/a_0$ in a manner similar to that of the experiments of Figs. 1, 2 and 3, and applying the least square method. The slope corresponds to the coefficient c_3 of Eq. (2), and therefore the reciprocal of its value can be regarded to be equal to the value of k_4, assuming Mechanism A. The values of k_9 and k_1 were not determined, since the values corresponding to the coefficients c_1 and $c_2$could not be obtained accurately.

	x_0	$e/v'_{\max}$	slope	k_4	
					mean
	M	sec.	sec. M	sec.$^{-1}M^{-1}$	
TP-A_1	7.64×10^{-4} 3.64×10^{-4}	0.20 0.20	6.14×10^{-5} 6.56×10^{-5}	1.63×10^{4} 1.52×10^{4}	1.6×10^{4}
TP-A_2	7.64×10^{-4} 3.64×10^{-4}	0.005 0.010	1.43×10^{-5} 1.52×10^{-5}	7.09×10^{4} 6.69×10^{4}	6.9×10^{4}
TP-D	9.13×10^{-4} 7.64×10^{-4} 3.64×10^{-4}	0.0124 0.0129 0.0143	1.54×10^{-5} 1.46×10^{-5} 1.17×10^{-5}	6.51×10^{4} 6.83×10^{4} 8.55×10^{4}	7.3×10^{4}
HRP1)					2.0×10^{4}

1) ref. (*4*)

$$E + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_I \qquad \text{Eq. (3)}$$

$$ES_I + AH \xrightarrow{k_7} ES_{II} + A \qquad \text{Eq. (4)}$$

$$ES_{II} + AH \xrightarrow{k_4} E + A + 2H_2O \qquad \text{Eq. (5)},$$

where E is the free molecule of HRP, ES_I Complex I, ES_{II} Complex II, AH and A the molecule of hydrogen donor in the reduced and oxidized form, respectively. Stationary state kinetics based on the above scheme leads to the following relationship as shown by Chance and Maehly (*9*)*,

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* More exactly, the relationship should be written as follows, though it was not mentioned explicitly by Chance and Maehly:

$$\frac{e}{v_0} = \frac{1}{k_1x_0} + \left(\frac{1}{k_4} + \frac{1}{k_7}\right) \times \frac{1}{a_0} + \frac{k_2}{k_1k_7} \times \frac{1}{a_0x_0}$$

However, this equation reduces to Eq. (6), since $k_7 \gg k_4$ and $\frac{k_2}{k_1k_7} \times \frac{1}{a_0x_0}$ is negligibly small under the experimental conditions.

$$\frac{e}{v_0}=\frac{1}{k_1x_0}+\frac{1}{k_4a_0} \qquad \text{Eq. (6)},$$

v_0, x_0 and a_0 being the same as those described above. Namely, in the case of HRP, there is not such an absolute term as found in Eq. (2).

In the case of the enzymes here used, however, the absolute term, c_1, was found to exist in the relationship between e/v_0, $1/x_0$ and $1/a_0$. Taking this term into account and based on the experimental results, the following two mechanisms are proposed as probable ones.

Mechanism A—This mechanism proposed contains an assumption of inhibition caused by the reversible combination of Complex I and Complex II with excess hydrogen donor as expressed by the following equation*:

$$ES_{I}+AH \underset{k_{-i}^{(1)}}{\overset{k_{i}^{(1)}}{\rightleftharpoons}} \overset{AH}{ES_{I}} \qquad \text{Eq. (7)}$$

$$ES_{II}+AH \underset{k_{-i}^{(2)}}{\overset{k_{i}^{(2)}}{\rightleftharpoons}} \overset{AH}{ES_{II}} \qquad \text{Eq. (8)},$$

where $\overset{AH}{ES_{I}}$ and $\overset{AH}{ES_{II}}$ represent the assumed inactive complexes produced by the combination of ES_{I} and ES_{II} with AH, respectively. The notation k assigned to each formula means the rate constant of each reaction. The following relationship** is deduced from Eqs. (3), (4), (5) and (7) and/or (8):

$$\frac{e}{v_0}=\left(\frac{1}{k_7}\times\frac{k_{i}^{(1)}}{k_{-i}^{(1)}}+/\text{or }\frac{1}{k_4}\times\frac{k_{i}^{(2)}}{k_{-i}^{(2)}}\right)+\frac{1}{k_1x_0}+\left(\frac{1}{k_7}+\frac{1}{k_4}\right)\times\frac{1}{a_0} \qquad \text{Eq. (9)}.$$

Mechanism B—The following mechanism of enzyme action is proposed for the second examination:

$$E+H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES_{I} \qquad \text{Eq. (3)}$$

$$ES_{I}+AH \xrightarrow{k_7} ES_{II}+A \qquad \text{Eq. (4)***}$$

$$ES_{II}+AH \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} ES_{II}\text{-}AH \qquad \text{Eq. (10)}$$

$$ES_{II}\text{-}AH \xrightarrow{k_9} E+A+2H_2O \qquad \text{Eq. (11)}.$$

In this formulation, it is suggested that ES_{II} reacts with AH to form an active "ternary complex" ES_{II}-AH. The notation k assigned to each formula represents the rate constant of each reaction. From these equations, the following relationship* is deduced:

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* Either one of these equations alone is enough to be assumed, as may be seen in Eq. (9).

** More precisely, the term $\frac{k_2}{k_1k_7}\times\frac{1}{a_0x_0}$ must be added to the end of the equation. However, the term may be disregarded for the same reason as that described in footnote of p. 799.

*** In this equation also, it might be taken that a ternary complex is formed intermedially as in the case of the reaction of the Complex II with AH. However, in view of the fact that the rate of this reaction is far greater than that of the reaction of Complex II with AH, its expression as the second-order reaction may be permitted.

$$\frac{e}{v_0}=\frac{1}{k_9}+\frac{1}{k_1x_0}+\left(\frac{1}{k_7}+\frac{k_9+k_{-8}}{k_8k_9}\right)\times\frac{1}{a_0} \qquad \text{Eq. (12)}$$

It cannot be decided only on the basis of the present experimental results which mechanism is more probable. However, it is clear that the reciprocal of c_2 of Eq. (2) is equal to k_1, whichever mechanism is assumed. The values of k_1 thus compluted are listed in Table I, in comparison with that of HRP obtained by Chance (*10*).

On the other hand, it is easily found from Eqs. (9), (12) and (2) that c_3 of Eq. (2) relates to the rate constants k_4 or k_8, k_{-8} and k_9 as shown in the following equations, taking into account the fact that Complex I reacts far more rapidly with hydrogen donor than Complex II does (*cf.* (*8*)):

assuming Mechanism A, $c_3=\frac{1}{k_4}$ Eq. (13)

assuming Mechanism B, $c_3=K_M\times\frac{1}{k_9}$ Eq. (14),

where K_M is a Michaelis constant in the usual sense, expressed as follows,

$$K_M=\frac{k_9+k_{-8}}{k_8} \qquad \text{Eq. (15).}$$

Thus, from the standpoint of Mechanism A, the value of rate constant k_4 can be easily obtained from the value of c_3 determined experimentally. The values of k_4 thus obtained are listed in Tables I and II. Comparison of these values with those of HRP reported by Chance (*10*) shows the following facts. The value of k_4 for guaiacol of TP-D is about thirty times greater than that of HRP, whereas those of TP-A_1 and TP-A_2 are considerably smaller than that of HRP. It has been already reported that the peroxidases extracted from various sources have different activities for hydrogen donors (*11*), but the results here obtained clearly shows that even in one kind of source there exist different kinds of peroxidases with different activities for a hydrogen donor. The values of k_4 for ascorbic acid, however, resemble each other, though more exactly, the following order is found: TP-D $\fallingdotseq$ TP-A_2 $>$ HRP $\fallingdotseq$ TP-A_1.

Assuming Mechanism B, the value of k_9 can be obtained as the reciprocal of c_1 of Eq. (2) and then the value of k_M can be computed using Eq. (14). The values obtained from the data of Fig. 1 are given in Table I. It will be of interest to examine spectrophotometrically whether the ternary complex is involved in the reaction. It may, however, be difficult to detect the ternary complex, since its absorption spectrum is not considered to show detectable difference from that of Complex II and, moreover, its lifetime is very small: the halflives of the ternary complexes of TP-A_1, TP-A_2 and TP-D are estimated to be 1.0, 0.8 and 0.2 millisecond, respectively, from the values of k_9. Thus, it is necessary to measure directly the rate of reaction between Complex II and hydrogen donor over a wide range of concentrations of the latter and to examine whether the reaction is of the second-order. Such an experiment was made by Chance (*4*) using Complex II of HRP and ascorbic acid, and it was concluded that this reaction is completely of the second-order and no ternary complex is formed in the reaction. He inferred also that similar results were obtained over smaller ranges of concentrations with other donor molecules (2), though it is not clear how high the concentrations of guaiacol used in his experiments were. From our results obtained by using guaiacol, however, the possibility is not denied that the ternary complex is formed in the reactions of our turnip peroxidases.

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* See the footnote** of p. 800.

SUMMARY

1. The kinetics of the over-all reaction of the purified turnip peroxidases A_1, A_2 and D has been studied using guaiacol and ascorbic acid as the hydrogen donor.

2. The reaction mechanisms of these enzymes were found to be essentially of the same nature as each other and as that of horseradish peroxidase, but the relationship between the reciprocal of the rate of over-all reaction and the reciprocals of the concentrations of hydrogen peroxide and hydrogen donor was different from that for horseradish peroxidase, only in that there is an additional absolute term (independent of the concentrations of hydrogen peroxide and hydrogen donor) in the case of the enzymes here studied.

3. This term was interpreted by two probable mechanisms, and the values of the rate constant of each reaction step were computed and compared with that of horseradish peroxidase.

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SOME PROPERTIES OF TAKA-AMYLASE A DEPENDING ON TEMPERATURE

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As reported in the previous paper (*1*, *2*), the optical rotatory power of taka-amylase A (TAA) was determined by authors for estimating the degree of folding of polypeptide chain in TAA molecule.

In parallel with these experiments, the effect of temperature on the optical rotatory power of TAA was studied and it was found that the optical rotation is constant below 30° and changes above this temperature. Other properties of TAA depending on temperature, the rate of enzymic reaction and liberation of amino acid from TAA (*3*), were also studied. The present paper deals with these temperature depending properties of TAA.

MATERIALS AND METHODS

TAA was purified and recrystallized two or three times by the method of Akabori *et al.* (*4*). The preparation was dissolved in distilled water. Dialyzed and urea denatured TAA were prepared by the method described in the previous paper (*2*).

Optical rotatory power of TAA was measured using D-line of a sodium lamp and denoted as $[\alpha]^t_D$, where t is temperature. Observation tube used is 20 cm. in length and covered with a mantle through which water of constant temperature circulated.

The enzymatic reaction was measured using a synthetic substrate, α-phenylmaltoside*** (*5*), instead of amylose.

The reaction rate was measured in the presence of sufficient amount of substrate in order that the reaction proceeds linearly with time. So the rate constant, k, is defined by the equation, $v=k\times e$, where v is reaction rate constant and e is the concentration of enzyme, k is indicated as moles of liberated phenol per minutes per μg. of TAA.

Determination of Liberated Amino Acid from TAA—TAA sample incubated at various temperature was taken up and divided in two parts. One was added with equal volume of 7 per cent solution of trichloroacetic acid and after the incubation for 20 minutes at 37°, the precipitate was filtered off. The filtrate was adjusted to pH 5.0 by adding N-NaOH and amino acid liberated was determined by the method of Moore and Stein

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*** α-phenylmaltoside is reported to be hydrolyzed by TAA (*5*) and excellent substrate for kinetic study because of its small molecular weight and well-defined structure.

(*6*).

The amount of liberated amino acid was indicated as leucine equivalent moles per mole of TAA assuming the molecular weight of TAA is 53,000 (*7*). The other part was used for the assay of enzymatic activity. It was measured by Fuwa's method (*8*) using amylose as the substrate at 37°.

RESULTS

1. Effect of Temperature on the Optical Rotatory Power of TAA—It was clearly observed that the optical rotatory power of aqueous solution of native TAA is about −12° and remains constant below about 30°. Above this temperaturature, however, the magnitude of the rotatory power decreases reversibly with the rise of temperature (Fig. 1a). In the case of urea denatured TAA, the rotatory power changes linearly in all the range of temperature studied and the degree of this change is much large than in native TAA (Fig. 1c).

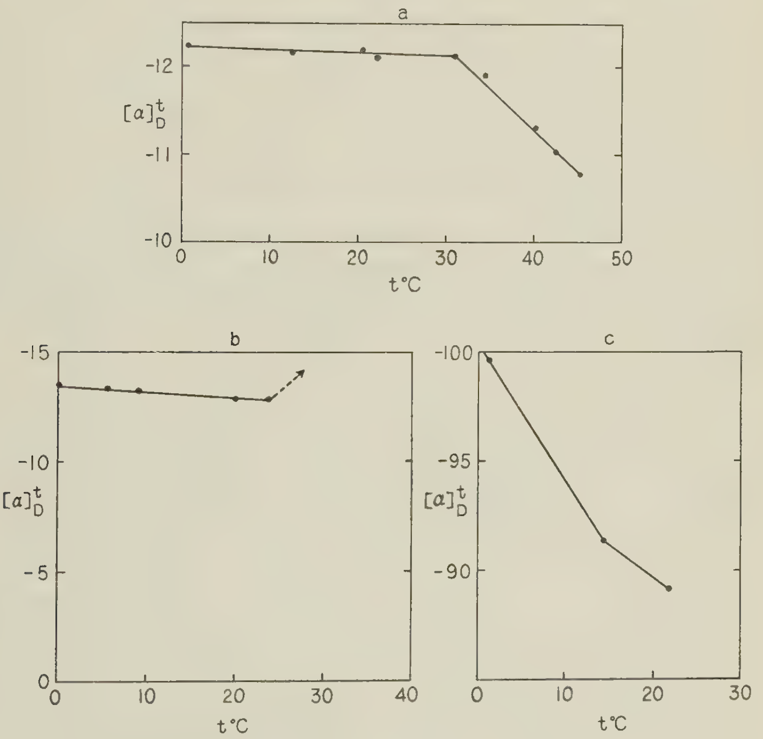

FIG. 1. Temperature dependence of optical rotatory power of native (a), dialyzed (b) and urea denatured TAA (c).

As reported previously (*1*, 2), the optical rotatory power of dialyzed TAA is practically identical with native TAA. However, when the temperature of its solution is elevated, the levo-rotation of dialyzed TAA decreases reversibly with the rise of temperature up to about 25°, and after heating above this temperature it cannot reverse by cooling (Fig. 1b).

2. *Rate of Catalytic Reaction*—The rate of hydrolysis of α-phenylmaltoside by TAA was determined at various temperatures. As can be seen in Fig. 2.

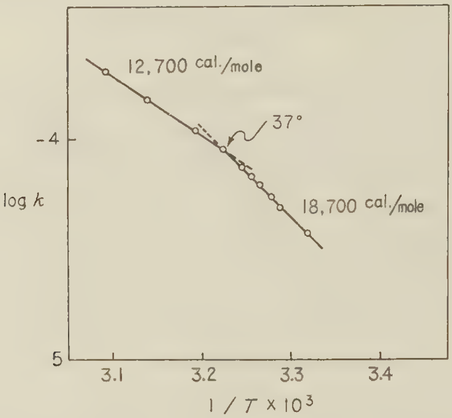

FIG. 2. Arrheniussian plot of reaction rate, k, for absolute temperature, T.

Arrheniussian plot of this reaction according to the equation, $\ln k = -\frac{E}{RT}$ (where T is absolute temperature, E is energy of activation and R is gas constant), shows two distinct lines crossing at 37°. The gradient of the line, the apparent energy of activation of the reaction, is smaller in the temperature range above 37° as compared with below 37°.

TABLE I

The Amount of Liberated Amino Acids from TAA during the Incubation of 42 Hours at Various Temperature and the Enzymatic Activity at the Time

Temperature (°C)	Liberated amino acid (Moles of leucine equivalent per TAA mole)	Enzymatic activity (per cent of initial value)
4	1.0	100
22	1.0	99
30	1.5	99
37	1.7	99
41	4.0	98
45	11.7	95

3. *Amino Acid Liberation from TAA*—It has been reported that an aqueous solution of TAA releases free amino acids at a slow rate (*3*). TAA dissolved in 0.1 *M* of veronal-HCl buffer of pH 7.0 was incubated at the various temperature and the rate of amino acid liberation was determined. Table I

shows the amount of liberated amino acid by 42 hours' incubation at various temperatures and the enzymatic activity at the end of incubation, which denoted as per cent of the initial value.

The significant amount of amino acid liberation was observed above 37°, but the liberation was extremely small below this temperature. During this incubation period practically no decrease of enzymatic activity was observed.

DISCUSSION

Fitts and Kirkwood (*9*) concluded from the theoretical ground that the optical rotatory power of a protein is a sum of two parts resulted from two distinct factors. One, the intrinsic residue rotation caused by the composing amino acids, the other, the rotation due to the helical structure of polypeptide chain, and this seems to be justified by Yang and Doty (*10*) from their experimental results. Destruction of helix causes the increase of levo-rotation. In our case, urea treated TAA decreases its rotatory power with the rise of temperature, and native TAA, though in smaller extent, has also the same relation above the temperature of about 30°. The similar results were attained by Harrington and Sela (*12*) on poly-L-proline II. This decrease of optical rotatory power is generally explained as the decrease of asymmetricity of the groups surrounding the asymmetric carbon atom in each amino acid residue by the increase of intramolecular thermal motion (*11*). Below this temperature, however, native TAA shows a constant rotatory power. This fact suggests that native TAA molecule holds its structure rigid below 30° resisting thermal agitation. The reversible change of rotatory power of native TAA at higher temperature region also seems to be restricted and probably different from the reversible change of rotation of urea denatured TAA. The inconsistency and irreversibility of rotatory power with temperature in dialyzed TAA suggest the role of calcium ion for the maintenance of the structure of the molecule.

It may be interesting to consider the fact mentioned above together with the fact that the liberation of amino acids from TAA is much more rapid in the temperature range above 37° than that below this temperature.

TAA has different energy of activation between above and below 37°. A similar phenomenon was observed in fumarase by Massay (*13*). He showed that the change of energy of activation corresponds to the temperature-dependence of the depolarizability of fluorescence-conjugated protein in acid solution. These facts might be caused by the change of molecular configuration of enzyme proteins accompanied with temperature change. The "less-rigid structure" is favorable to lower the barrier of energy of activation. More intensive investigation will be required for the precise explanation.

SUMMARY

1. Native, dialyzed and urea-denatured taka-amylase A (TAA) were compared each other in their temperature-dependence of optical rotatory

power. Urea-denatured TAA shows a negative temperature coefficient of this power. Native TAA also shows the same phenomenon above 30°, though lesser extent, and below this temperature, the rotatory power remains constant.

2. TAA has two different values of energy of activation for its catalytic action to a substrate, α-phenylmaltoside. The critical temperature of this difference is about 37°.

3. The rate of amino acid liberation from TAA also shows a distinct change at this temperature.

4. These facts were discussed in reference to the structure of this enzyme.

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SPECIFICITY OF HYDANTOINASES, DIHYDROPYRIMIDINASES AND DECARBAMYLASES

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According to recent studies, the biosynthesis of uracil proceeds from aspartic acid through successive formation of carbamyl aspartic acid (ureidosuccinic acid) (*1–4*) and dihydroorotic acid (*5–7*), accompanied by a side reaction of interchange between ureidosuccinic acid and hydantoin 5-acetic acid (*5*). On the other hand, decomposition of uracil is reported to start off with reduction (*8–22*) to dihydrouracil or oxidation to barbituric acid (*23–29*), both of which products are then hydrolyzed. In the cource of pyrimidine metabolism there are thus reversible opening of hydantoin and dihydropyrimidine rings. The present communication is concerned with the studies on the substrate specificity of the enzymes involved in those reactions as well as decarbamylation of ureido acids. To obtain the enzymes active enough for chemical assay, adaptation technique was applied and soil bacteria were cultivated in the presence of each of substrates as a sole N source.

EXPERIMENTALS

Synthesis of Substrates—The following substrates* were prepared according to the procedures already known or their slight modifications. L-Ureidosuccinic acid (US), L-hydantoin 5-acetic acid (HA), L-ureidoglutaric acid (UG), L-hydantoin 5-propionic acid (HP), potassium salt of carbamyl taurine, carbamyl L-asparagine (US-NH_2), anhydro carbamyl L-asparagine (HA-NH_2), L-dihydroorotic acid (DHO), dihydrouracil (DHU), carbamyl anthranilic acid, hydantoic acid, hydantoin, 1-methyl hydantoin, N-methyl hydantoic acid, DL-5-methylhydantoin, uracil (U), β-ureido propionic acid (BUP), isobarbituric acid (IBarb), orotic acid, DL-isoserine (ISer), dihydrothymine (DHT). The purity of each compound was checked by Kjeldahl-N estimation. The melting points coincided with those reported by previous workers, except US-NH_2 which melted at 159–161° in contrary to 137–138° (*30*).

Carbamyl DL-isoserine, CISer, was prepared from ISer, applying nitrourea method (*31*); prismatic crystal of m. p. 137° (uncorrected). 5-Hydroxy-dihydrouracil, 5OH-DHU was prepared as follows. CISer solution was acidified with hydrochloric acid and evaporated in a boiling water bath and the residue was recrystalized from water; plate crystal melting at 234° (uncorrected), higher than 228° of the preparation from 5-bromohydrouracil reported by Fischer (*32*). Barbituric acid (Barb), and other compounds used were commercial products.

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* Each abbreviation of the compounds used in this article are cited here.

Enzymes—Suspensions of bacteria isolated from soil near this laboratory were used. The growth liquid media contained 0.4 per cent hydantoin 5-acetic acid (HA), 0.4 per cent hydantoin 5-propionic acid (HP), 0.3 per cent uracil (U), or 0.3 per cent dihydrouracil (DHU), respectively, as N source material, 6 per cent glycerol, 0.2 per cent citric acid, 0.05 per cent KH_2PO_4, and 0.05 per cent $MgSO_4 \cdot 7H_2O$. The solution was adjusted to pH 7 with 10 per cent KOH. The bacteria which grow in media containing HA, HP, U or DHU are called HA-cells, HP-cells, U-cells and DHU-cells, respectively. They are all *Gram* negative rods. Inoculum solution was spread on the agar media of above composition containing respective substrate and after growth for about 18 hours at 27°, the cells were harvested by centrifugation, washed twice with 0.9 per cent NaCl, and suspended in 10 volumes of distilled water; 1 ml. suspension contained nearly 8 mg. bacteria, when desiccated. The growth of U-cells on the agar medium containing U as a sole N source was poor and required double growth time of others to obtain corresponding amount of bacterial mass. For deadaptation, each strain was cultured successively 4 times on the agar broth media. Autolysates of bacteria were prepared by leaving the cell suspension for 6 hours at 37° under toluene layer and dialysing overnight against tapping water.

Methods—Composition of the test solutions is as follows: 1 ml. 0.01 *M* substrate solution or water+3 ml. 0.1 *M* buffer+1 ml. enzyme solution. This was incubated 18 hours under toluene layer at 37°. NH_3 formed was determined colorimetrically by indophenol method (*33*) after diffusion in Conway's unit and the formation of 1 mole NH_3 from 1 mole substrate was termed 100 per cent. Determination of ureido compounds was as follows: the test solution was deproteinized by addition of equal volume of 10 per cent trichloroacetic adid and centrifuged at 4,000 r.p.m. for 30 minutes; taking 1 ml. supernatant solution, the chromogen substance was determined by the colorimetric method of Koritz and Cohen (*34*).

Developing solvent for ascending paper chromatography was upper layer of *n*-butanol, glacial acetic acid and water (4 : 1 : 3) mixture, and to detect the spots the ninhydrin reaction was used for amino acids and Fink's reaction (*11*) for dihydropyrimidines and ureido compounds.

RESULTS

I. Enzyme of the Bacteria Adapted to Hydantoin 5-Acetic Acid (HA-cells)

A. *Intact Cell Suspension*–This bacterial suspension decomposes HA, forming aspartic acid and NH_3. The optimal pH of this enzyme system was 7–8 as shown in Table I. The decomposition of HA shows that its hydantoin ring is opened by 5-carboxymethylhydantoinase (*5*) to produce US, which is further hydrolysed by ureidosuccinase (*35*) to aspartic acid and NH_3.

Table II shows the results when this cells act upon the several hydantoin bodies, dihydropyrimidines, and ureido compounds at pH 7.7. The quantity of NH_3 formed from US does not reach that to be judged from decreased ureido reaction, and much less when the presence of aspartase in this bacteria would be considered. This means a partial closure of US, though it does not indicate whether the product was DHO or HA. DHO can be decomposed by the HA-cells to aspartic acid with production of NH_3, indicating the presence of dihydroorotase. DHU was also subjected to ring opening, but not to NH_3 formation; therefore, BUP can not be decarbamylated by the HA-cells.

TABLE I

Optimal pH of Hydrolysis of Hydantoin 5-Acetic Acid by the Bacteria Adapted to Hydantoin 5-Acetic Acid (HA-cells)

pH	7	8	9	10
NH_3 formed	58%	62%	53%	29%

The reaction system contained 1 ml. 0.01 *M* substrate or water, 3 ml. 0.1 *M* buffer, 1 ml. cell suspension (1 : 10) under toluene layer. Glycine-phosphate mixture (0.1 *M* glycine solution in 0.1 *M* Na_2HPO_4) was adjusted with 0.1 *N* HCl or 0.1 *N* NaOH to the required pH. Time 18 hours, and temperature 37°.

TABLE II

Hydrolysis of Hydantoin Bodies, Dihydroorotic Acid, and Ureido Compounds by the Bacteria Adapted to Hydantoin 5-Acetic Acid (HA-cells)

Substrates	NH_3 formed		Change of ureido reaction		Detected by paper chromatography
	I	II1)	I	II1)	
L-Dihydroorotic acid (DHO)	125%				Asp (+)
L-Hydantoin 5-acetic acid (HA)	164%				Asp (+)
L-Ureidosuccinic acid (US)		42%		−69%	Asp (+)
L-Aspartic acid (Asp)		88%			
L-Asparagine (Asp-NH_2)		188%			
Anhydro carbamyl L-Asparagine (HA-NH_2)		<5%		+0%	Asp (−) Asp-NH_2 (−)
Carbamyl L-Asparagine (US-NH_2)		14%			Asp (−) Asp-NH_2 (−)
L-Hydantoin 5-propionic acid (HP)	0%		+0%		
L-Ureidoglutaric acid (UG)		0%		−0%	

1) Experiment carried out on another day.

The reaction condition was as in Table I. 0.1 *M* phosphate buffer (pH 7.7) was used.

This strain has high asparaginase action. However, it carried out much less NH_3 formation from HA-NH_2 and US-NH_2 than from HA and US, and showed no increase of an ureido reaction positive substance from HA-NH_2.

It is worth while to remark that HP and UG, homologs of HA and US, are neither hydrolyzable nor interconversible by HA-cells.

The rings of hydantoin, 1-methyl hydantoin and 5-methyl hydantoin remained intact and no NH_3 production was observed from hydantoic acid, N-methyl hydantoic acid, carbamyl taurine or carbamyl anthranilic acid.

B. Heated Suspension and Dialysed Autolysate of HA-cells—After heating the bacterial suspension at 55° for 30 minutes or after autolysis, the following four enzymes, namely dihydroorotase, 5-carboxymethylhydantoinase, dihydrouracilase and asparaginase remain intact. However, by these treatments, ureidosuccinase and aspartase are abolished.

C. Deadapted HA-cells—After deadaptation and even after autolysis, were dihydroorotase, dihydrouracilase, and asparaginase proved to be present, whereas 5-carboxymethylhydantoinase, ureidosuccinase, and aspartase were no more found. Accordingly the latter three enzymes which have participated during growth in the utilization of HA belong to adaptive enzyme, while the former three enzymes, namely dihydroorotase, dihydrouracilase, and asparaginase seem to be constitutive one, that is to say, the reversible reaction between DHO and US is carried out by a constitutive enzyme, whereas decarbamylation of US becomes positive only after adaptation.

II. Enzymes of the Bacteria Adapted to Hydantoin 5-Propionic Acid (HP-cells)

A. Intact Cell Suspension—This strain produces NH_3 from HP and UG, but not from glutamic acid under the conditions tested. Optimal pH of HP decomposition is near 8 (Table III).

Table III

Optimal pH of Hydantoin 5-Propionic Acid Decomposition by the Bacteria Adapted to Hydantoin 5-Propionic Acid (HP-cells)

pH	6	7	8	8.6
NH_3 formed	0%	30%	48%	45%
Change of ureido reaction	0%	+20%	+31%	+28%

The reaction condition was as in Table I. 0.1 *M* phosphate buffer for pH 6–8 and glycine-phosphate mixture for pH 8.6 were used.

Table IV shows that HP-cells catalyse the reversible conversion HP$\rightleftarrows$UG. Besides 5-carboxyethylhydantoinase and ureidoglutarase, HP-cells contain dihydroorotase, dihydrouracilase, and asparaginase as in the case of HA-cells, but as expected, none of 5-carboxymethylhydantoinase, ureidosuccinase, and aspartase is present which were found in HA-cells.

B. Heated HP-cell suspension—5-Carboxyethylhydantoinase disappears on heat treatment, but ureidoglutarase remains intact. The latter enzyme has optimal pH at 9 (Table V).

C. Deadapted HP-cells—In this case, both 5-carboxyethylhydantoinase and ureidoglutarase disappear; in other words, these enzymes belong to adaptive enzyme, whereas dihydroorotase and dihydrouracilase remain as the constitutive enzyme just as in the case of HA-cells.

Table IV

Hydrolysis of Hydantoin Bodies, Dihydroorotic Acid and Ureido Compounds by the Bacteria Adapted to Hydantoin 5-Propionic Acid (HP-cells)

Substrates	NH_3 formed		Change of ureido reaction	
	I	II[1]	I	II[1]
L-Dihydroorotic acid (DHO)		0%		+48%
L-Hydantoin 5-acetic acid (HA)		0%		+ 0%
L-Ureidosuccinic acid (US)		0%		−23%
L-Aspartic acid (Asp)		0%		
L-Asparagine ($Asp\text{-}NH_2$)	97%			
Anhydro carbamyl L-asparagine ($HA\text{-}NH_2$)		0%		+ 0%
Carbamyl L-asparagine ($US\text{-}NH_2$)	15%			
L-Hydantoin 5-propionic acid (HP)		48%		+30%
L-Ureidoglutaric acid (UG)	49%		−59%	
L-Glutamic acid (Glu)		0%		
Dihydrouracil (DHU)		0%		

1) Experiment carried out on another day.

The reaction condition was same as in Table I. pH was 8; 0.1 *M* phosphate buffer was used.

Table V

Optimal pH of Ureidoglutarase

pH	7	8	9	10
NH_3 formed	7%	40%	97%	53%

The reaction mixture contained 1 ml. 0.01 *M* UG or water, 3 ml. 0.1 *M* buffer and 1 ml. HP-cell suspension (1 : 10) which is previously heated at 55° for 30 minutes. pH 7 and 8, phosphate buffer; pH 9 and 10, glycine NaOH buffer.

III. Enzymes of the Bacteria Adapted to Dihydrouracil (DHU-cells)

A. Intact Cell Suspension—The most NH_3 formation from DHU by this strain was observed at pH 8 (Table VI). At this pH (Table VII), no NH_3 was produced from U, but existence of dihydrouracilase, dihydroorotase and BUP-decarbamylase was shown.

B. Deadapted DHU-cell suspension—In this case, hydrolysis of BUP is no more observed, but ring opening of DHO as well as of DHU is carried out.

Table VI

Optimal pH of Dihydrouracil (DHU) Hydrolysis by the Bacteria Adapted to Dihydrouracil (DHU-cells)

pH	6	7	8	9
NH_3 formed	4%	12%	19%	11%

The reaction system contained 1 ml. 0.01 *M* DHU or water, 3 ml. 0.1 *M* buffer and 1 ml. DHU-cell suspension (1 : 20). pH 6–8, phosphate buffer; pH 9, glycine-phosphate mixture (see Table I).

Table VII

Hydrolysis of Pyrimidine Bodies and Ureido Compounds by the Bacteria Adapted to Dihydrouracil (DHU-cells)

Substrates	NH_3 formed	Change of ureido reaction	Detected by paper chromatography[1]	
			Fink's method	Ninhydrin
Uracil (U)	0%		(−)	(−)
Dihydrouracil (DHU)	91%		BUP (+)	β-Ala (+)
β-Ureidopropionic acid (BUP)	78%		DHU (+) BUP (+)	β-Ala (+)
L-Dihydroorotic acid (DHO)	0%	+43%		
L-Ureidosuccinic acid (US)	0%	−19%		
β-Alanine (β-Ala)	0%			

1) R_f values. DHU, 0.63; BUP, 0.77: β-Ala, 0.44.

The reaction system contained 1 ml. 0.01 *M* substrate or water, 3 ml. 0.1 *M* phosphate buffer (pH 8) and 1 ml. cell suspension (1 : 10). This was incubated at 37° for 18 hours under toluene layer.

IV. Enzymes of the Bacteria Adapted to Uracil (U-cells)

A. Intact Cell Suspension—Judging from NH_3 formation, optimal pH of U decomposition is 7 regardless of presence or absence of toluene addition (Table VIII). From unknown grounds the chromatographic demonstration

Table VIII

Optimal pH of Uracil (U) Hydrolysis

Toluene Overlaying	pH	5.3	6	7	8	9
−	NH_3 formed	12%	21%	25%	9%	8%
+		5%	5%	12%	9%	5%

The reaction system contained 1 ml. 0.01 *M* U or water, 3 ml. 0.1 *M* buffer and 1 ml. U-cell suspension (1 : 10) in the presence of toluene or (1 : 20) in its abscence. pH 5.3–8, phosphate buffer; pH 9, glycine-phosphate mixture (see Table I).

of amino acids eventually produced was negative.

The results of tests at pH 7 with other substrates under toluene layer (Table IX) show at first the presence of dihydrouracilase and dihydroorotase. Ureidosuccininase which has been found to be an adaptive enzyme is not detected, whereas BUP is decomposed as it was by DHU-cells. This indicates that during growth of soil bacteria using U as N source material, reduction of U should have taken place to produce DHU which was successively hydrolysed to BUP, inducing the formation of BUP decarbamylase.

TABLE IX

Hydrolysis of Pyrimidine Bodies and Their Related Compounds by the Bacteria Adapted to Uracil (U-cells)

Substrates	NH_3 formed	Change of ureido reaction	Detected by paper chromatography[1]	
			Fink's method	Ninhydrin
Uracil (U)	10%		(−)	(−)
Dihydrouracil (DHU)	30%[1]		DHU (+) BUP (+)	β-Ala (+)
β-Ureidopropionic acid (BUP)	17%[1]		DHU (±) BUP (+)	β-Ala (+)
β-Alanine (β-Ala)	0%			
Barbituric acid (Barb)	0%			
Isobarbituric acid (IBarb)	23%			
L-Dihydroorotic acid (DHO)	0%	+35%		
L-Ureidosuccinic acid (US)	0%	−21%		

1) Experiment carried out on another day.

The reaction condition was similar as in Table I. 0.1 *M* phosphate buffer (pH 7) was used.

U-cells do not produce NH_3 from Barb, but do from IBarb.

B. Deadapted U-cells—Dihydrouracilase and dihydroorotase which have been proved as constitutive remain intact also in this case. However, NH_3 formation from U and IBarb becomes undetectable.

V. Hydrolysis of 5-Hydroxy-dihydrouracil (5-OH-DHU)

The observation of IBarb decomposition by U-cells lead the author to carry the test with 5-OH-DHU. Table X indicates that hydrolysis of 5-OH-DHU by intact U-cell suspension proceeds as follows: 5-OH-DHU → ClSer → ISer. After deadaptation from U, the enzyme action 5-OH-DHU → ClSer remains, but the decarbamylation of ClSer to ISer is abolished. It is also the case with the heated suspension of adapted U-cells. Accordingly, 5-hydroxy-dihydrouracilase (5-OH-DHU → ClSer) belongs to a constitutive enzyme, while ClSer decarbamylase is an adaptive enzyme.

The experiments described in this paper were carried out stepwise during two years or more, being often interrupted due to preparation of various substrate compounds.

Since decarbamylation of BUP and CISer was carried out by the soil bacteria previously adapted to U, some experiments were undertaken to determine whether the formation of CISer-decarbamylase would take place in the case of adaptation to DHU and also whether BUP and CISer would be decarbamylated by a common enzyme. The strain newly isolated and adapted to DHU was tested and found to be able to produce NH_3 from CISer and even from DHT (Table XI). CISer hydrolysis was observed also in the case of adaptation to BUP. Decarbamylation of CISer was, however, abolished after deadaptation.

TABLE X

Hydrolysis of 5-Hydroxy-dihydrouracil and its Hydrolysed Products by the Bacteria Adapted to Uracil (U-cells)

Substrates	NH_3 formed	Detected by paper chromatography[1)	
		Fink's method	Ninhydrin
5-Hydroxydihydrouracil (5-OH-DHU)	50%	5-OH-DHU (−) CISer (+)	ISer (+)
Carbamyl-DL-isoserine (CISer)	55%	5-OH-DHU (−) CISer (+)	ISer (+)
DL-Isoserine (ISer)	0%		ISer (+)

1) R_f values. 5-OH-DHU, 0.52; CISer, 0.40; ISer, 0.31.

The reaction condition was as in Table IX.

TABLE XI

Hydrolysis of Carbamyl DL-Isoserine and Dihydrothymine by the Bacteria Adapted to Dihydrouracil (DHU-cells)

Substrates	NH_3 formed	
	I	II[1)
β-Ureidopropionic acid (BUP)	70%	
Carbamyl DL-isoserine (CISer)	57%	
Dihydrouracil (DHU)		75%
Dihydrothymine (DHT)		50%

1) Experiment carried out on another day.

The reaction condition was similar as in Table VII.

DISCUSSION

The results mentioned above concern with three groups of enzymic reactions; the ring rupture of hydantoins, that of dihydropyrimidines and the decarbamylation of ureido compounds.

The enzymes of soil bacteria for opening of hydantoin ring, for instance, of HA and HP are of adaptive nature and so far as studied, one substrate induces formation of one enzyme specific for it. 5-Carboxymethylhydantoinase is proved to be different from 5-carboxyethyl hydantoinase. Therefore, side chain length seems to determine the specificity of various hydantoinases.

Dihydropyrimidines used at first as substrates in the present paper are DHO, DHU and 5-OH-DHU. The heterocyclic rings of these compounds were all reversibly opened by constitutive enzymes of soil bacteria. Dihydroorotase which was found at first by Kornberg (*5*) in *Zymobacterium oroticum* grown in orotate containing medium was reported afterwards by Yates and Pardee (*36*) to be a constitutive enzyme. DHT is now found to be decomposed under NH_3 production by bacteria adapted to DHU. Whether or not the hydrolytic ring-opening of various dihydropyrimidines is attributed to a single enzyme has not been investigated this time. Grisolia *et al.* (*19*) reported that the activities in calf liver of DHU and DHT hydrolysis could not be separated from each other even after manifold purification. On the other hand, according to a recent report of Campbell (*22*) a specific dihydrouracilase free from enzymes for other dihydropyrimidines could be prepared from *Clostridium uracilicum.*

The specificity of decarbamylating enzymes seems to be determined by C number of amino acid moiety. Hydrolysis of US and UG are carried out by enzymes different from each other. Also different from these ureidosuccinase and ureidoglutarase is the enzyme for hydrolysis of BUP. Mechanism of formation of this enzyme in the case of adaptation to U is possibly due to intermediary formation of BUP from U.

As to the hydrolysability of CISer by the soil bacteria adapted to U, a similar possibility seems to be assumed, namely that U would have been hydrated by growing bacteria to 5-OH-DHU, followed by its ring-rupture to CISer, which might induce the formation of an enzyme active for itself. It would be favorable to fortify this assumption if a rigid specificity of CISer-decarbamylating enzyme could be manifested. The experiments carried out resulted in that this enzyme and even the hydrolytic enzyme for DHT were found in the cells adapted to DHU as well as to BUP. A single enzyme, which is different from ureidosuccinase and ureidoglutarase, seems to be effective to the carbamylated β-amino acids of C_3 chain length, regardless of the presence of hydroxyl or methyl group in α-position. Such a concept is visible also in the report of Grisolia *et al.* (*21*). Thus, the positive decarbamylation of CISer does not serve as a proof for possible preceeding of hydration of U. However, the result that enzymic NH_3 production from IBarb, but not from Barb, was shown solely by the bacteria adapted to U and never in any case of adaptation to other substrates, inclusive of DHU, may indicate that there is a pathway of hydration of U to 5-OH-DHU which is then metabolized either by direct hydrolysis or after dehydrogenation to IBarb. Then this would be a third pathway of U decomposition in addition to the well known pathways, reduction to DHU and oxidation to Barb.

SUMMARY

Specificity of the hydrolytic enzymes for several hydantoin compounds, dihydropyrimidines and ureido acids was investigated in view of their adaptive formation in soil bacteria.

The enzymes for the reversible opening of hydantoin rings are of adaptive nature and their specificity is determined by the length of side chains of the substrates. Hydantoin acetic acid and hydantoin propionic acid are hydrolyzed by enzymes different from each other.

So far as studied with dihydrouracil, dihydroorotic acid, 5-hydroxy-dihydrouracil and dihydrothymine, those dihydropyrimidines are decomposed to corresponding carbamyl amino acids by an enzyme system of constitutive nature.

The decarbamylating enzymes for ureido acids are produced by adaptation and their substrate specificity for ureido acids depends upon the difference of amino acid moiety. Hydrolysis of carbamyl aspartic acid and carbamyl glutamic acid is carried out by ureidosuccinase and ureidoglutarase, respectively. However, carbamyl β-alanine, carbamyl isoserine and β-ureidoisobutyric acid are hydrolyzed by a single enzyme. Therefore, substitution at α-carbon of β-alanine moiety, may it be by hydroxyl or methyl group, does not effect the specific combination of the enzyme with the β-ureido acid of C_3 chain length. The soil bacteria grown on media containing uracil as a sole nitrogen source produced ammonia from isobarbituric acid, but not from barbituric acid. A possible hydration of uracil as one of metabolic pathways was discussed.

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STUDIES ON THE RESPIRATORY ENZYME SYSTEMS OF PLANTS

I. ENZYMATIC OXIDATION OF α-NAPHTHYLAMINE IN RICE PLANT ROOT

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It is very important from the standpoint of the agronomical technique to known the general activity of plant root, and there have been measured the activities of enzymes such as cytochrome oxidase, peroxidase *etc*. and the activities of enzyme systems such as respiratory rate, TTC*-reducing rate *etc*., which were appraised as the activity-diagnosing methods.

Nomoto and Ishikawa (*1*), Yamazaki (*2*), Sakai and Yoshida (*3*), and Yamada and Oota (*4*) have also pointed that the α-naphthylamine* (α-NA)-oxidizing activity may be available for a technique to diagnose the activity of root, and it seems to be one of the useful methods. For instance, it was shown that this activity has a high correlation to the respiratory activity of rice plants which are cultured in various conditions (*4*). The α-NA-oxidizing activity, however, will be meaningless if the biochemical mechanism of this oxidation is not made clear. It has been supposed in prior studies (*3, 4*) that hydrogen peroxide takes part in this oxidation, but detailed and quantitative experiments about this problem have not been reported.

This paper presents the results that α-NA is oxidized by peroxidase in the presence of H_2O_2, and that the α-NA-oxidizing activity from the agronomical standpoint does not depend upon the peroxidase activity itself but correlates closely with the rate of production and supply of H_2O_2 or other peroxides in the tissues to be measured.

EXPERIMENTALS

Rice Plant—Noorin No. 29 in the 5th leaf-age was used.

Homogenate—2.9 g. of rice plant roots were homogenized with sand in mortar, into which 30 ml. of 0.1 *M* phosphate buffer, pH 6.0, were added. The homogenate was centrifuged at $1{,}000 \times g$ for 10 minutes. The turbid supernatant is termed as the homogenate in the following description.

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* Abbreviations: α-NA, α-naphthylamine; TTC, triphenyltetrazoriumchloride; DPNH, reduced diphosphopyridine nucleotide; and TPNH, reduced triphosphopyridine nucleotide.

Partially Purified Enzymes—Glycolic acid oxidase was prepared from rice plant leaf by the method of Chiba, Kawai and Ueda (*5*).

Peroxidase was purified to the final step of the first fractional precipitation with ammonium sulphate in the method of Morita and Kameda (*6*) from the root of water-cultured rice plant in ripening stage.

Crystalline Peroxidase—It was kindly supplied from Dr. Morita. It was prepared from Japanese raddish and named JRP-a by him (*6*).

Assay of α-NA—One* is a colorimetric method by diazo reagent. Two ml. of the sample containing α-NA less than 5 γ were introduced into a 20 ml. messflask containing 10 ml. of water, and 1 ml. of 1 per cent sulfanilic acid solution in 20 per cent acetic acid and 1 ml. of 0.1 per cent $NaNO_2$ aqueous solution were added, and then it was filled up to 20 ml. with water. After 30 minutes, the optical density at 524 mμ was measured.

Another method depends upon the fact that the minimum in absorption spectrum of α-NA at 260 mμ rises up to an apprecible extent during its oxidation with enzyme and H_2O_2. Change in absorption spectrum is shown in Fig. 1. As shown in this figure, the spectrum of the oxidized product in the visible range is so flat that we can not use it for the assay of α-NA as in the method using guajacol.

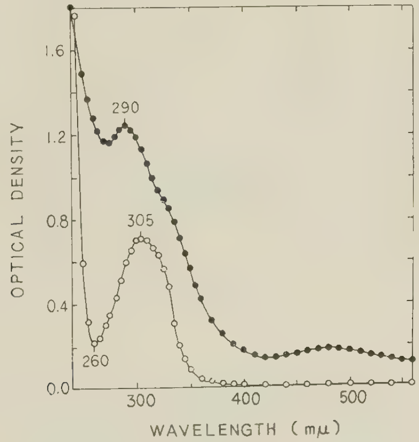

Fig. 1. Absorption spectra of solutions of α-NA, and its oxidized product.

—○—, 10 ppm α-NA; —●—, oxidized α-NA with partially purified peroxidase and 2.49 ppm H_2O_2. Molar concentrations of α-NA and H_2O_2 are the same.

These measurements of the reaction at ultra violet region were carried out using Hitachi spectrophotometer model EPU-2 with quartz cubets. The change in optical density at 260 mμ was recorded.

RESULTS

Decrease of α-NA by Rice Plant Root—When root of rice plant was immersed into a solution containing α-NA, the concentration of α-NA decreased little by little as shown in Fig. 2. If we use the material plant from which the stem (and leaf) was cut off at the height of 2 cm. from the boundary between top and root, the decreasing rate of the concentration of α-NA became more slowly.

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* Unpublished method, Yamada, N. and Oota, Y.

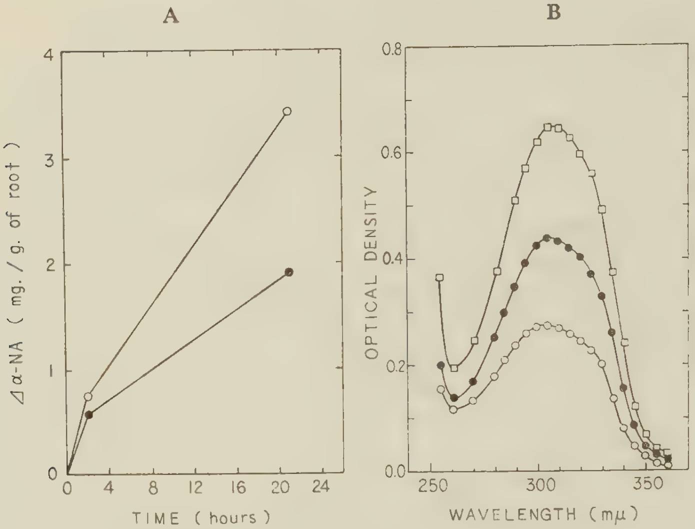

FIG. 2. Decrease of α-NA by intact root.

In A, the root having (○), and not having (●) their top parts (stem and leaf). Initial concentration of α-NA, 20 ppm.; temperature, about 20°; each plot is the mean value of data in three jars containing five rice plants, respectively.

B shows absorption spectra of solution at initial (□), and final (21 hours after) having (○) and not having (●) top parts. Each spectrum is an example of three jars, respectively.

As shown above, the α-NA-oxidizing activity corresponds to such biological treatment as the detachment of top part of plant.

Considering the change in the spectra of the solutions in ultraviolet region, a majority of the oxidized product will be precipitated in the cells of root and it is difficult to go out to the solution, and therefore the root is discolored to dark violet.

Necessity of H_2O_2 for α-NA-Oxidation—When we use intact root, the decolorization of root by α-NA needs a long time, but the decolorization becomes very deep in a few minutes with the addition of a small amount of H_2O_2. In both cases described above, the developed colors of the roots are the same.

In Fig. 3., partially purified glycolic acid oxidase from rice plant leaf, which has also peroxidase activity tested with guajacol as hydrogen donor, is used. As shown in the figure, α-NA oxidation does not proceed with only the enzymes and α-NA, but as soon as H_2O_2 is added to the reaction mixture, the oxidation proceeds.

As glycolic acid oxidase is a flavine enzyme which oxidizes glycolic acid to glyoxylic acid and produces H_2O_2 (*5*, *7*), α-NA-oxidation by peroxidase

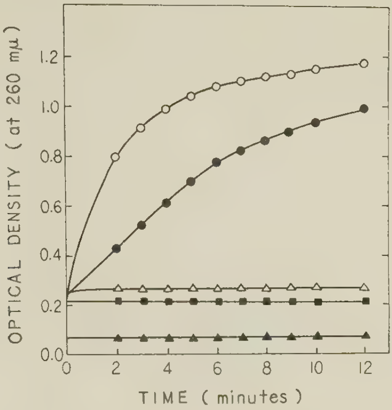

FIG. 3. Effect of H_2O_2 upon peroxidase-catalysed α-NA oxidation. —○— 0.5 ml. crude enzymes+200 γ α-NA+500 γ H_2O_2, —●— 0.5 ml. crude enzymes+200 γ α-NA+100 μM Na-glycolate, —△— 0.5 ml. crude enzymes+200 γ α-NA, —■— No enzyme+200 γ α-NA+500 γ H_2O_2, —▲— 0.5 ml. crude enzymes+500 γ H_2O_2. Total volume, 10 ml.; temperature, 23°; crude enzymes prepared from rice plant leaf contain peroxidase and glycolic acid oxidase.

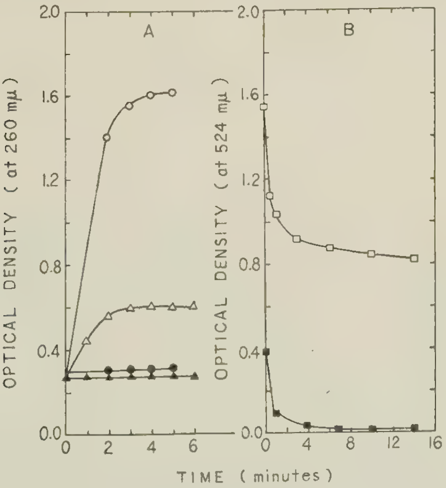

FIG. 4. α-NA-oxidation by crystalline peroxidase.

A shows the increase of oxidized product: (○, ●) contain 10 times more peroxidase than (△, ▲). (○, △) contain 100 γ H_2O_2 and 2.0 μM α-NA, and (●, ▲) only α-NA.

B shows the decrease of α-NA: α-NA, 4.0 μM (□), 1.0 μM (■); and H_2O_2, 10 γ.

can proceed rapidly using the H_2O_2 produced by glycolic acid oxidase, when we add glycolic acid to the reaction mixture containing the enzyme and α-NA.

Partially purified preparation of peroxidase from rice plant root can also oxidize α-NA only in the presenae of H_2O_2, but the addition of glycolic acid does not effect to this system because there is no glycolic acid oxidase activity in root of rice plant.

The crystalline peroxidase from Japanese raddish can also oxidize α-NA only in the presence of H_2O_2 as well as in the foresaid examples, as shown in Fig. 4.

From these facts, it will be possible that α-NA-oxidation demands the presence of H_2O_2.

Inhibitory Experiment—α-NA-oxidation with partially purified peroxidase was inhibited by sodium azide as shown in Fig. 5.

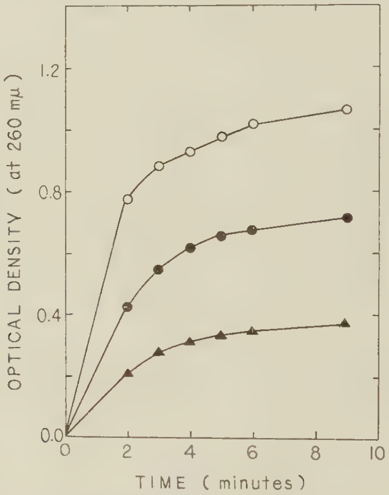

FIG. 5. Inhibition of α-NA-oxidation by sodium azide.

Final concentration; α-NA, 20ppm.; H_2O_2 50ppm.; NaN_3, none (○), 1×10^3 *M* (●), and 1×10^2 *M* (▲). Temperature; 20°.

Stoichiometry of α-NA-oxidation—When we observe the α-NA-oxidation as a subcellular reaction, it is shown that the enzyme is subjected to self-inhibition. In other words, the reaction rate becomes very slow in a few minutes even without any inhibitor. The inhibited enzyme can not more act upon guajacol. This inhibition may be done by H_2O_2 or reaction products, and the mechanism of this self-inhibition will be remained to be a problem in future.

Although there is such self-inhibition, if we use excess enzyme and α-NA and a smaller amount of H_2O_2, we can determine the quantity of H_2O_2

necessary for the oxidation of one mole of α-NA.

From the data shown in Fig. 6., it seems probable that H_2O_2 and a-NA react in equimolecular amounts apparently.

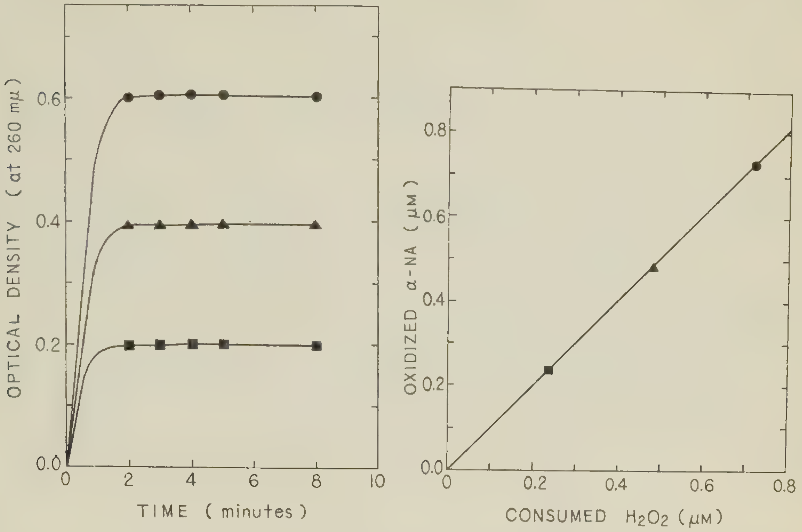

Fig. 6. Stoichiometry of α-NA-oxidation.

Reaction mixture contained: α-NA, 1.4 μM; H_2O_2, 0.72 μM (●), 0.48 μM (▲), 0.24 μM (■); and 1.0 ml. of partially purified peroxidase from rice plant root. Total volume, 10 ml.

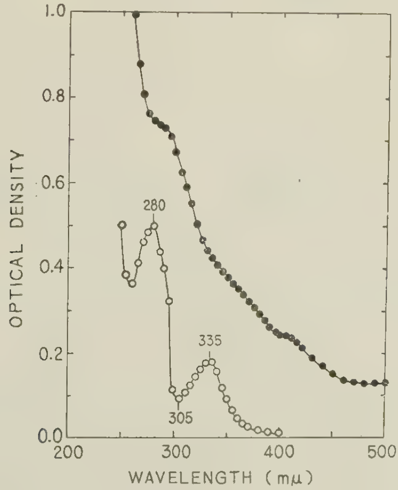

Fig. 7. Absorption spectra of solution of β-NA and its oxidized product.

—○—, β-NA; —●—, its oxidized product by partially purified peroxidase with H_2O_2.

Reaction with β-NA and Benzidine—Partially purified peroxidase prepared from rice plant root acts upon β-NA only in the presence of H_2O_2, probably as well as upon α-NA. The change in absorption spectrum in ultraviolet region of β-NA with peroxidase and H_2O_2 is shown in Fig. 7. Perhaps this reaction will produce an analogous product to the of α-NA, but the color of it is slightly different and is as dark brown as the oxidized product of guajacol. This preparation of peroxidase acts also upon benzidine (*p*-diaminodiphenyl) with H_2O_2, and makes it discolored to brown passing through bluish green.

Comparison of α-NA-Oxidation Activity of Intact Root with That of Homogenate Preparation—As shown in Fig. 8., with 2.9 g. of intact root, the change in optical density at 524 mμ which shows the amount of α-NA by means of colorimetry, was about 0.30 per 2 ml. of the solution (100 ml. in total), in which the roots were immersed, for 6 hours at 30°.

The homogenate prepared from 2.9 g. of root cultured as well as in the former showed 0.28 of optical density change per 1 ml. of homogenate solution, for 2 minutes. The volume of homogenate solution was 30 ml. in total.

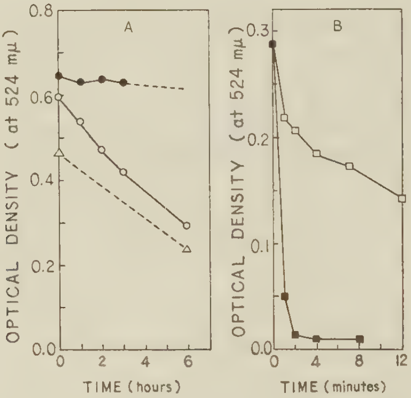

FIG. 8. Comparison of the α-NA-oxidation by intact root with that by the homogenate preparation.

A. Intact root: —●—, without root; —○—, with root; and —△—, change in optical density at 305 mμ. (the peak point in absorption spectrum of α-NA).

B. Homogenate preparation: —□— contained 10-fold-diluted enzyme of original homogenate(—■—).

After the calculation of the change in optical density per unit fresh weight of root and unit time, it was found that the homogenate preparation system supplied with H_2O_2 has more than one thousand times of α-NA-oxidizing activity compared with that of the intact root system. When we consider the efficiency of the extraction, the inactivation during the extraction, and a fact that the reaction in the homogenate preparation system is measured at about 17°, it will be clear that the ratio of the activity of homogenate system to that of intact root system becomes larger. Then we may conclude that the amount of peroxidase is too sufficient in root cells to support the α-NA-oxidation in the intact roots.

DISCUSSION

What is the player of α-NA-oxidation or the rate limiting factor in the oxidation? There will be three possibilities about them as follows:

1. The amount of enzyme concerning to the α-NA-oxidation.
2. Assuming the enzyme to be peroxidase, the rate of production and supply of H_2O_2 or peroxide which can be used by peroxidase.
3. The penetration rate of a-NA into the root cells.

It is certainly suggested that the α-NA-oxidizing reaction should be held by peroxidase from the data in Figs. 3, 4 and 5. The author would not except the possibility of the existence of the aromatic hydroxylase which was found in mammalian liver and coupled DPNH (*8*, *9*) or TPNH (*10*, *11*), but the homogenate preparation itself has scarcely α-NA-oxidizing activity.

In addition, there was sufficient amount of peroxidase in the cells of rice plant root as shown in Fig. 8. Tagawa and Shin reported that there are much peroxidase in wheat germ compared with the cytochrome components (*12*). As shown in Fig. 9., the author also found the presence of much

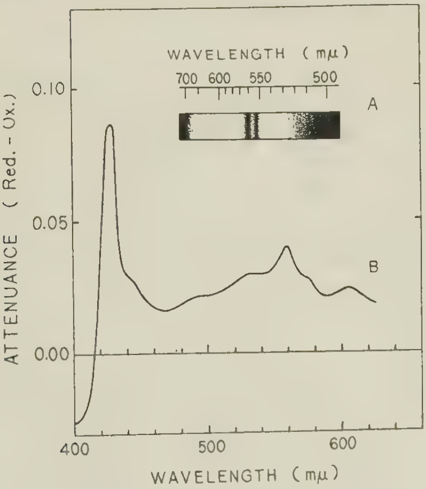

FIG. 9. Hemoproteins in rice plant root.

A. Absorption bands of young root tip paste by means of handspectroscope.

B. Difference spectrum of mitochondrial preparation of rice plant root.

Reduction was carried out with $Na_2S_2O_4$.

amount of the components which have the absorption maximum adjacent to 560 mμ after sodium-hydrosulfite-reduction and are considered to be peroxidase or cytochrome b members in the mitochondrial preparation of rice plant root by means of handspectroscope method and in the intact root tip by the opal glass method*.

Aimi and Fujimaki (*13*) reported that, when they made the slice of rice plant root and added α-NA and H_2O_2 to them, the outer three cell layers became very deep violet and the discolorization of the other cells was not so significant. If we insert at first the root into a solution containing α-NA and H_2O_2 and then slice it, it is found that the outer three layers become very deep violet as well as in the former case. From these observations, as far as in the cell region chiefly concerned to α-NA-oxidation, the penetration rate of α-NA is sufficiently high.

Consequently, although it is not perfectly denied that α-NA is oxidized by other enzyme system than peroxidase, but generally speaking, peroxidase plays a major part in this oxidation. Additionally, the amount of peroxidase is sufficient in root cells. Then, it seems that the 2nd possibility is most probable.

The fact that the decolorization of the root only with α-NA needs a long time while the decolorization with α-NA and H_2O_2 proceeds very rapidly, would support the foresaid conclusion.

The classification of the hydrogen-donor to peroxidase by Yamazaki (*14*) showed that aniline belongs to the XH_2 group in which it can hardly lose two electrons at the same time during oxidation and two moles of it may react with one mole of H_2O_2 as shown in following equations.

$$2XH_2+H_2O_2=(XH)_2+2H_2O$$
$$YH_2+H_2O_2=\ \ Y\ \ +2H_2O$$

As the structure of α-NA is analogous to that of aniline, the author expected that α-NA would belong to this type or XH_2 type in view of H_2O_2 consumption. On the contrary, it has been shown that one mole of of H_2O_2 reacted with one mole of α-NA and α-NA seems to belong to the YH_2 group which can lose two electrons at the same time and consumes equimolecular H_2O_2 as itself. In this case, although it may be supposed that the basal structure of the product is α-imino-naphthalene radical or the first product (may be $(XH)_2$) will be oxidized by one more mole of H_2O_2, the mechanism of the oxidation and the chemical structure of the oxidized products will be cleared in the forthcoming studies. The product obtained in this enzyme system was a dark violet material which was soluble scarcely in water, slightly in ether, and easily in ethylacetate.

The production of H_2O_2 in the plant cell may be done by glycolic acid oxidase in leaf blade and leaf sheath, but unfortunately no activity of this enzyme is found in root. Accordingly, the survery of such flavine enzyme or other H_2O_2-donating enzymes in root is a very important work in future. Tagawa and Shin considered the H_2O_2 production by DPNH oxidase in wheat germ and the oxidation of DPNH by this H_2O_2 and peroxidase (*12*).

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* The data about cytochromes of rice plant will be reported soon in details.

There may be hydrogen-donors belong to YH_2 group in rice plant root. And if they may be oxidized directly with oxygen and peroxidase, they will produce H_2O_2 and become a source of H_2O_2.

SUMMARY

1. The partially purified peroxidase from leaf and root of rice plant respectively and crystalline peroxidase named JRP-a from Japanese raddish can oxidize α-NA with H_2O_2. The oxidized product of α-NA was a dark violet colored substance, and was soluble scarcely in water, slightly in ethyl ether and very easily in ethylacetate. β-NA was also oxidized by this enzyme system as well as α-NA.

2. The peroxidatic oxidation of α-NA needs one mole of H_2O_2 per one mole of α-NA.

3. The α-NA-oxidizing activity which seems to be available for the method of diagnosing of the root activity in agronomy, may represent the rate of the production and supply of H_2O_2 or other peroxides in tissue to be measured.

I wish to express my thanks to Dr. Y. Morita for supplying the crystalline peroxidase used in this study, and also to Dr. I. Yamazaki for his helpful suggestions.

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The Journal of Biochemistry, Vol. 47, No. 6, 1960

STUDIES ON TAKA-MALTASE

I. PURIFICATION AND PROPERTIES OF TAKA-MALTASE FROM TAKA-DIASTASE

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(Received for publication, January 19, 1960)

The maltose hydrolysing enzyme in molds has once been studied in detail from the viewpoint of the enzyme specificity for the comparison to α-glucosidase in other sources (*1*, *2*), and lately the mechanism of maltose-transglucosylation has come under investigation (*3*, *4*). However, the purification of maltase was not yet schemed but a few attempts (*5*, *6*). Since there are two kinds of enzymes in molds which have maltase activity—the one is saccharogenic amylase and the other α-glucosidase, in other term transglucosylase – these enzymes must be at first isolated in pure state in order to clarify their specificities and properties.

As to the carbohydrases in taka-diastase, Taka-amylase A (α-amylase) was already crystallized (*7*) and Taka-amylase B (saccharogenic amylase) was also highly purified (*8*), therefore the properties of these two enzymes were fully studied. For a further study on the starch-saccharogenic enzyme system in taka-diastase, α-glucosidase, the third carbohydrase, should be separated in pure state from the other enzymes in taka-diastase.

Some α-glucosidases in molds such as those in *Aspergillus oryzae* (*9*) and in *Aspergillus niger* (*10*) were already separated in crystalline form from culture media, but Taka-maltase in taka-diastase was not isolated yet. The author attempted to separate Taka-maltase from the discarded solution in the crystallization course of Taka-amylase A from taka-diastase (*7*). For this purpose, the column chromatography was applied, using the weak anion exchange resin, Duolite A-2.

METHODS

Maltase Activity—One ml. of 0.04 per cent maltose solution was added to the mixture, which consisted of 1.0 ml. of the enzyme solution and 1.0 ml. of 0.5 *M* acetate buffer solution of pH 4.7. After 10 minutes incubation at 30°, the reaction mixture was mixed with 3 ml. of Somogyi's reagent (*11*) to stop its enzyme action and heated for 30 minutes in a boiling water bath. To the cooled solution, 2 ml. of Nelson's reagent (*12*) was added and agitated mildly. The resulted blue solution was diluted, after a lapse of 20 minutes, to 50 ml. with water. The optical density of the solution was measured spectrophotometrically at 500 mμ with 10 mm. glass cell.

α-Glucosidase Activity—Similar to the above, the reaction mixture was made by mixing 1.0 ml. of the enzyme solution, 1.0 ml. of 0.5 *M* acetate buffer solution of pH 5.0 and 1.0 ml. of 0.125 per cent α-phenyl-D-glucoside solution. The reaction mixture was incubated at 30°, for 30 minutes and added with 3 ml. of 5 per cent sodium carbonate solution to stop the enzyme action. Mixed with 1.0 ml. of the two-fold diluted Folin's phenol reagent (*13*), the reaction mixture was kept at 30° for 30 minutes. The optical density of the solution was measured with spectrophotometer at 660 mμ with 10 mm. glass cell.

Amylase Activity—Amylase activity was determined by Fuwa's method (*14*), measuring its saccharogenic activity.

To express the enzyme activity, an enzyme unit was set by adopting the optical density measured as mentioned above, and the specific enzyme activity was expressed by the enzyme unit per mg. of protein nitrogen (U/P. N.) or the enzyme unit per optical density at 280 mμ (U/E_{280}). Protein nitrogen was estimated by the ordinary micro-Kjeldahl method.

EXPERIMENTS AND RESULTS

Purification—The supernatant of the rivanol fractionation, which was usually discarded in the purification course of Taka-amylase A, was used in this experiment as the starting material for the separation of Taka-maltase. The ammonium sulphate was added to the supernatant to bring it to a half saturation, and amylase was adsorbed on starch by the filtration through potato starch-celite (1 : 1 by weight) on a Büchner funnel. To the filtrate ammonium sulphate was added again to 0.8-saturation. The precipitate was dissolved in water after filtration and dialysed against water, using a collodion bag, overnight in a cold room. The dialysed fluid was added with calcium acetate to the final concentration of 0.05 *M* and added with acetone to 40 per cent concentration. The precipitate obtained by centrifugation was dissolved in a small amount of 0.05 *M* acetate buffer solution of pH 5.0 and dialysed against the same buffer solution in a cold room. The method of Taka-maltase purification mentioned above is shown in Scheme 1.

An overall purification to *ca.* 33-fold was achieved with about 2.6 per cent recovery of α-glucosidase. Table I shows summaries of the results of purification in terms of recovery, the total and specific enzymatic activity at each stage of purification.

Column Chromatography—The dialysed solution was applied to a 2×30 cm. column of Duolite A-2 (acetate form, 100 to 180 mesh), which had been previously equilibrated with 0.05 *M* acetate buffer solution of pH 5.0. Eluting with this buffer solution, increasing its molar concentration stepwisely, the effluent was collected in 5 ml. fractions. On every fraction, protein content was measured through spectrophotometry at 280 mμ and activities of α-glucosidase, maltase and amylase were determined. As shown in the chromatogram of Taka-maltase (Fig. 1), the effluent with 0.1 *M* buffer solution had two peaks which indicated maltase activity. These enzymes were designated as Taka-maltase I and II, and their specific activities were different as shown in Table II.

Detailed and critical analyses on the homogenity of enzymes could not be undertaken because of the scanty amount of the enzyme protein isolated.

SCHEME. 1

Purification Procedure of Taka-maltase

Taka-diastase Sankyo 2 kg.
extraction with 10 liter of water, filtration
Filtrate I
addition of 0.25 *M* Ca-acetate in an equal volume of the filtrate, centrifugation
Supernatant II
0.75 saturation with $(NH_4)_2SO_4$, centrifugation
Precipitate
dissolved in water and dialysed, ...III
addition of 1% rivanol solution in volume of 0.04 of the dialysed fluid, filtration
Filtrate
addition of 1% rivanol solution in volume of 0.06 of the filtrate, centrifugation
Precipitate — Supernatant IV
Precipitate → Crystalline Taka-amylase A
Supernatant: a half saturation with $(NH_4)_2SO_4$, filtration through potato starch—celite (1 : 1)
Filtrate
0.8 saturation with $(NH_4)_2SO_4$, filtration
Precipitate
dissolved in water and dialysed, ...V
addition of Ca-acetate to 0.05 *M*, addition of acetone to 40% concentration, centrifugation
Precipitate
dissolved in 0.05 *M* acetate buffer of pH 5.0, ...VI
Duolite A-2 column chromatography
Purified Taka-maltase

TABLE I

Specific Activities and Yields of Preparation at Each Stage of Purification

Fraction number in Scheme 1	α-Glucosidase activity				Maltase activity			
	Total units	Yield (%)	U/P.N.	U/E_{280}	Total units	Yield (%)	U/P.N.	U/E_{280}
I	295000	100	10	0.25	114000	100	4.0	0.10
II	201000	68	—	0.21	92800	81	—	0.097
III	66000	22	4.7	0.22	29200	26	2.1	0.098
IV	48800	17	8.4	—	20000	18	3.5	—
V	11600	3.9	110	1.9	4800	4.2	46	0.80
VI	7600	2.6	330	8.8	3000	2.6	130	3.5

Properties—Both Taka-maltase I and II hydrolysed maltose and α-phenyl-D-glucoside, but very weakly α-methyl-D-glucoside, while neither one hydrolysed amylose and sucrose. When maltose was applied in a high concentration, these enzymes exhibited the transglucosylation activity. Details on the hydrolysis of α-D-glucosides and α-maltosides will be published in the succeeding paper.

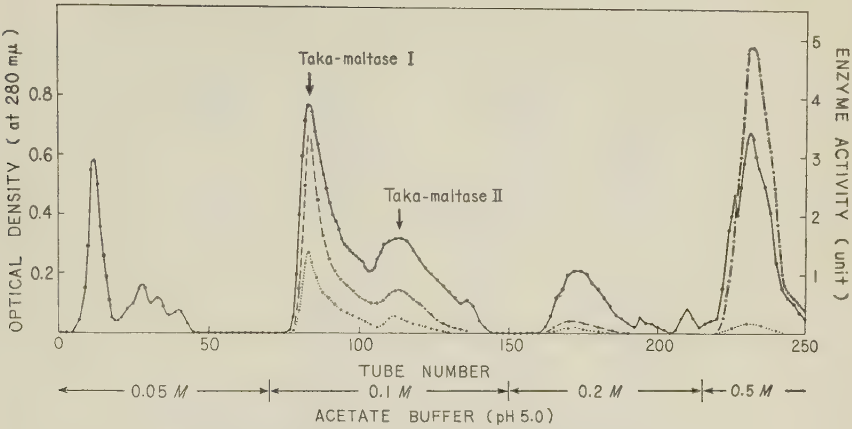

FIG. 1. Column chromatogram of Taka-maltase on Duolite A-2.
—— : Protein content (optical density at 280 mμ);
- - - -: α-glucosidase activity;
· · · · · : maltase activity;
-·-·-: amylase activity.

TABLE II

Specific Activites of Purified Taka-maltases

	α-Glucosidase activity		Maltase activity	
	U/Total-N1)	U/E_{280}	U/Total-N1)	U/E_{280}
Taka-maltase I	860	40	270	15
Taka-maltase II	—	20	—	10

1) Quantity of total nitrogen was used instead of protein nitrogen because of insufficient quantity of the samples.

The rates of maltose and α-phenyl-D-glucoside hydrolysis by Taka-maltase I and II were studied as a function of pH with McIlvain's citric acid—sodium phosphate buffer. No significant difference was observed between Taka-maltase I and II in their pH-activity curves as shown in Fig. 2.

The effect of pH on the stability of the enzymes in case of incubation at 37° for one hour is shown in Fig. 3. The patterns of the pH-stability of the enzymes were similar with each other, except that Taka-maltase II had a broader stable pH-region as compared with Taka-maltase I.

In order to know the stability against heat, the change in enzyme activity was measured on the enzyme solutions which were incubated at pH 5.3 (acetate buffer) for various lengths of time at various temperatures as shown in Fig. 4. In general, Taka-maltase II seemed to be a little more stable than Taka-maltase I. Both enzymes were very stable below 30°, somewhat unstable over 30° and rapidly inactivated at the temperature over 40°.

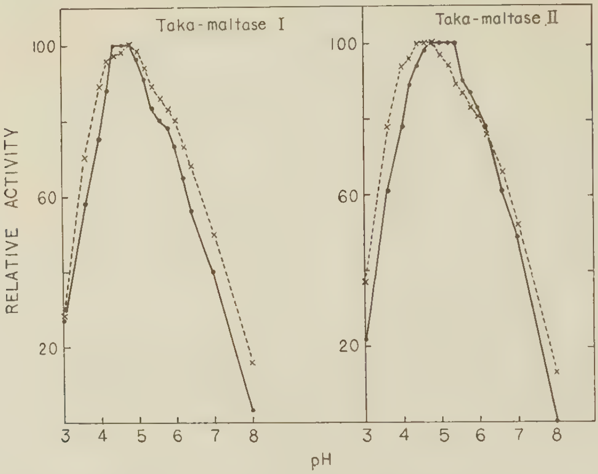

FIG. 2. pH-Activity curves of Taka-maltase I and II
—●—: Maltase activity; --×--: α-glucosidase activity.

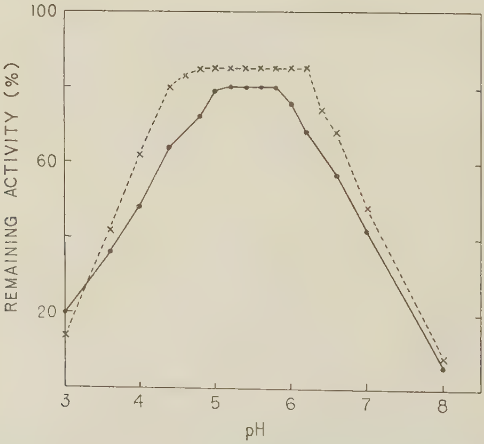

FIG. 3. The effect of pH on the stability of Taka-maltase I and II at 37°.
—●—: Taka-maltase I; --×--: Taka-maltase II.

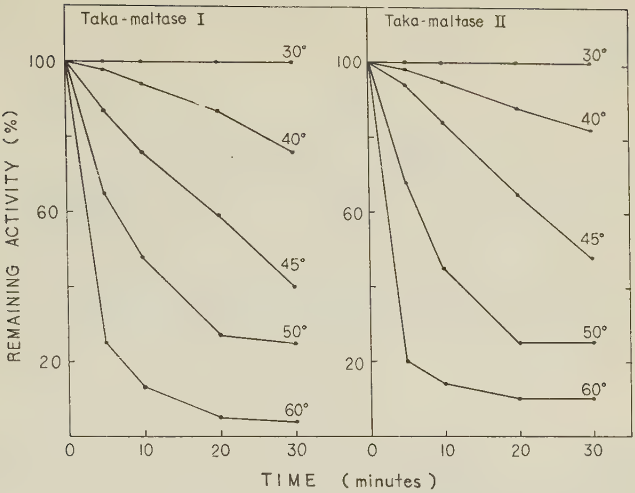

FIG. 4. The rate of inactivation at different temperatures.

DISCUSSION

Although many studies (*1*) on the maltase activity of taka-diastase were reported, they dealt with a combined activity of saccharogenic amylase and α-glucosidase. Taka-amylase A (*7*) and Taka-amylase B (*8*) were isolated in 1951 and 1953 respectively, and the latter was shown to have maltase and amyloglucosidase activities. Crystallization of amylase-free maltase from *Aspergillus oryzae* was recently reported by Sugawara *et al.* (*9*), but the whole starch-saccharogenic enzyme system is not yet entirely clear.

In the present study it is found by the column chromatography that Taka-maltase consists of two components, Taka-maltase I and II. Certain differences between the two enzymes are found in the specific activity, the pH stability and the heat stability, while no difference is found in the substrate specificity. Judging from these results, the two maltases may be considered to be different proteins but with the same active site.

It is a well known fact that the crystalline ribonuclease shows two components on the chromatogram obtained through the ion exchanger column, due to the slight variation in number of free carboxyl groups notwithstanding the same enzymatic activity (*15*, *16*). From these viewpoints, there are possibilities that Taka-maltase has two components, the one being derived from the other due to some modifications in its composition which might have occurred during the purification procedure, or that Taka-maltase I and II are originally produced as two different enzymes by *Aspergillus oryzae*.

According to the report of Sugawara *et al.* (*9*), the maltase obtained by them from a culture medium of *Aspergillus oryzae* has pH-optimum at 3.4 and temperature-optimum at 50°. Taka-maltase obtained by the present author has different properties with regard to pH- and temperature-optimum and precipitability. These facts probably depend on the difference of the strain of *Aspergillus oryzae* used.

SUMMARY

Two maltases (α-glucosidases), Taka-maltase I and II, were separated from taka-diastase, by means of column chromatography using weak anion exchange resin, Duolite A-2. Their properties were determined and discussed.

The author wishes to express his deep gratitude to Prof. S. Akabori for his kind guidance and to Dr. T. Ikenaka for his valuable advice throughout the investigation, and also to Sankyo Co., Ltd. for their kind supply of 'Takadiastase Sankyo.'

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MECHANISM OF CYANIDE RESISTANCE IN ACHROMOBACTER

III. NATURE OF TERMINAL ELECTRON TRANSPORT SYSTEM AND ITS SENSITIVITY TO CYANIDE

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(Received for publication, January 20, 1960)

In previous papers (*1*, *2*), it was reported that the strain of *Achromobacter* isolated from soil was found to become cyanide resistant in its respiration, when it was grown in the presence of cyanide, and that the mechanism of the cyanide resistance could be explained by the remarkable increase of the amount of cytochrome a_2 in the resistant cells. To make this mechanism more clear, it is necessary to study about the nature of terminal electron transport system of these bacteria especially with regard to the physiological significance of cytochrome a_2 and its sensitivity to cyanide. However, little has been known on the terminal electron transport system of the bacteria belonging to the genus *Achromobacter*. The bacteria used in this study were similar to *Escherichia coli* or *Aerobacter aerogenes* in their cytochrome spectrum. These strains of bacteria were reported to contain cytochrome b_1, a_1 and a_2, and cytochrome a_2 had been thought to have a function of a cytochrome oxidase. However, even in these bacteria, many evidences which threw doubt on the physiological significance of cytochrome a_2 as a cytochrome oxidase, have been presented (*3*, *4*), and the role of these cytochromes in the terminal electron transport system has never been made clear.

The object of the present paper is to make clear the mechanism of terminal electron transfer in the bacteria especially with regard to the role of cytochrome a_2 and its sensitivity to cyanide. Studies were carried out by using a water soluble cytochrome preparation obtained from a subcellular particulate preparation by extracting with deoxycholate.

EXPERIMENTALS

Cultivation of Bacteria—*Achromobacter* strain 7 used in the previous studies (*1*, *2*) was cultivated in 50 liters of nutrient broth (pH 7.0) containing 1 per cent meat extract, 1 per cent polypeptone and 0.5 per cent solium chloride. The cultivation was carried out at 30° with continuous stirring and air bubbling (60 liters of air per minute). The inoculum size of the bacteria was about 5×10^7 cells per ml. of the broth, and they were harvested when numbers of cells were reached to 2×10^9 per ml. of the broth. The cultivation time was about 12 hours. The rate of the bacterial growth in this case was much slower than that carried out under very aerobic condition. This was due to the low oxygen tension in the medium caused by the low efficiency of the apparatus in dissolving oxygen into the medium. Therefore, the cultivation was found to be carried out under fairly anaerobic condition. Thus, 250 g. of bacteria were obtained and were collected by centrifugation and washed once with saline.

Soluble Cytochrome Preparation—To 250 g. of wet cell was added 150 ml. of $M/15$-phosphate buffer (pH 7.4). This thick suspension of cells was exposed to the sonic oscillation (10 KC) for 15 minutes. The intact cells and cell debris in the sonicate were centrifuged down at $5000 \times g$ for 20 minutes. The turbid supernatant solution was further centrifuged using Spinco Model L ultracentrifuge at $100{,}000 \times g$ for 2 hours, and the yellowish supernatant solution was removed off. To the dark reddish precipitate (subcellular particulate preparation) thus obtained, was added an equal volume of 2 per cent solution of deoxycholate and the pH of the mixed solution was adjusted to 7.0 with concentrated solution of sodium hydroxide. After standing for 24 hours at 2° under aerobic condition, the solution was centrifuged at $145{,}000 \times g$ for one hour. The clear russet supernatant solution obtained was used in this study.

Measurement of Oxido-reduction Level of Cytochromes in Preparation—The oxido-reduction level of cytochrome b_1 and cytochrome a_2 in the preparation was estimated by measuring the change of the difference between the optical density at 561 mμ and that at 545 mμ, and the difference between the optical density at 630 mμ and that at 650 mμ, respectively. The reduction of the cytochromes in the preparation was carreied out by adding succinate or DPNH under anaerobic condition. The anaerobic condition was made in Tunberg type photocell by evaccuating air or by covering the sample with liquid parafin.

Preparation of Haemin—Haemins of cytochrome b and a_2 were prepared from the subcellular particulate preparation according to the method of Barrett (*5*). The well washed particulate preparation which had no catalase activity was dispersed in a Waring Blender with sufficient water, acetone and HCl to give 100 ml. of suspension containing 30 ml. of water, 70 ml. of acetone and 0.35 g. of HCl. After standing for a minute, the residue was separated from the acid-acetone solution by centrifugation. An equal volume of ether was added to the supernatant immediately after separation, and the ethereal solution of haemin obtained was washed with 1 per cent HCl until free from actone. The ether was removed *in vaccuo* from the solution of haemins and the oily residue was dissolved into a small volume of acetone. After removing insoluble substances by centrifugation, a small volume of acetone solution of haemins was dissolved into a mixture of pyridine and 0.2 N NaOH (1 : 2). Both ether solution and pyridine-NaOH solution were used for spectrophotometric experiment.

Materials—DPN used in this experiment was the sample of Nutritional Biochemicals Coporation. Reduced DPN (DPNH) was formed from DPN by reducing with alcohol and alcohol dehydrogenase.

RESULTS

Properties of Subcellular Particulate Preparation—The absorption spectrum of the particulate preparation (reduced form) was shown in Fig. 1. It was noticeable that the absorption peak around 630 mμ corresponding to the α-band of reduced cytochrome a_2 was much higher than that prepared from the cell grown under aerobic condition (*1*). It was also found that the oxidation of DPNH or succinate by tne particulate preparation was rather insensitive to cyanide, whereas the oxidation activity of the preparation obtained from the cells grown under aerobic condition was almost completely inhibited by cyanide (*1*). These facts agreed with the findings in the previous paper (*1*) that the increase in the amount of cytochrome a_2 caused the decrease of the sensitivity of the respiration to cyanide. However, the precise study of this point will be reported in the next paper (*6*).

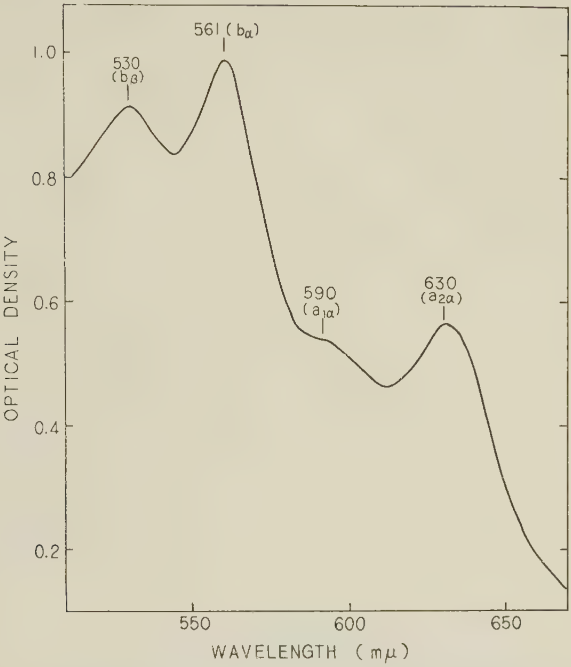

FIG. 1. Absorption spectrum of particulate preparation reduced with dithionite.

Numbers in the figure show the wave length (mμ) of the point, and letters in the parenthesis show the type of cytochrome corresponding to the peak.

Particulate preparation was suspended in *M*/15-phosphate buffer (pH 7.4) and about 10 mg. of solid dithionite was added in 4 ml. of the suspension just before the measurement. Absorption spectrum was measured using Cary Model 14 Spectrophotometer based on the opal glasss method (*9*).

Properties of Soluble Cytochrome Preparation—The absorption spectrum of the preparation was shown in Fig. 2. The all cytochrome components found in the particulate preparation were extracted as oxidized form. This preparation possessed weak dehydrogenase activities for DPNH and succinate, when methylene blue was used as the electron acceptor. The all cytochromes in the preparation were slowly reduced under anaerobic condition in the presence of DPNH or succinate as the electron donor. However the separation of these cytochromes could not be successful by fractionation with ammonium sulfate, with ultracentrifugation or chromatography on cation exchange resin (XE-64). In addition, all cytochromes in tne preparation were salted out from the ammonium sulfate solution of less than 20 per cent saturation, while they were slowly precipitated by ultracentrifugation (145,000 × g, for several hours).

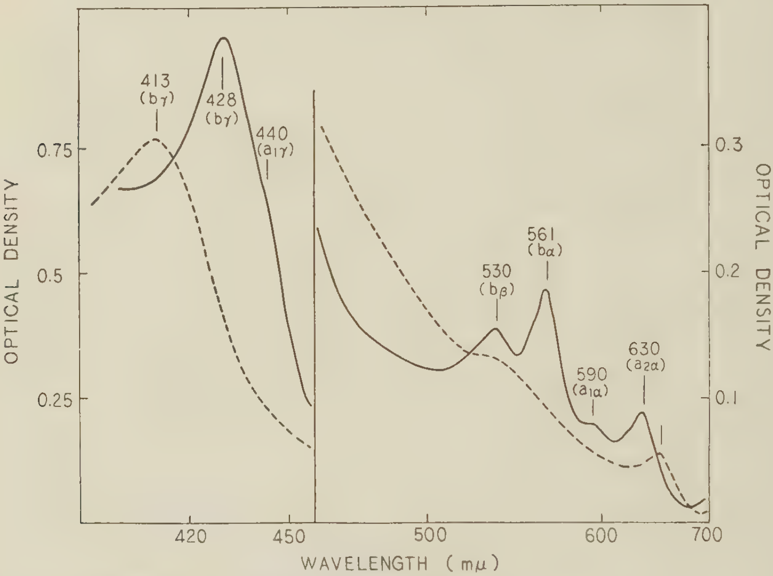

FIG. 2. Absorption spectrum of soluble preparation.
Broken line; oxidized. Solid line; reduced with dithionite.
Numbers in the figure show wave length (mμ) of the point and letters in the parenthesis show the type of cytochrome corresponding to the peak. Spectrum was measured using Beckman DK-2 Spectrophotometer.

Though the combination of the cytochromes and cyanide were also studied spectrophotometrically using the preparation, any shift of absorption maxima by the addition of cyanide (10^{-2} M) could not be observed on both oxidized and reduced forms of cytochrome b and a_2.

Effect of Cyanide on Reduction of Cytochrome b and a_2 in Soluble Preparation—When succinate was used as electron donor, the cytochromes in the soluble preparation were gradually reduced under anaerobic condition as shown in Fig. 3. It was interesting that the reduction of cytochrome b seemed to occur after that the reduction of cytochrome a_2 was fairly proceeded. In the presence of cyanide, though the lag period in the reduction of cytochrome b was rather prolonged, the rate of the reduction was as fast as that in the absence of cyanide. On the other hand, the reduction of cytochrome a_2 was remarbably inhibited by cyanide. It was important, however, that the partial reduction of cytochrome a_2 could be observed even in the presence of 10^{-3} M of cyanide. With regard to cytochrome a_1, precise study could not be carried out owing to the faint absorption spectrum of the cytochrome in the preparation. The similar results were also obtained when DPNH was used as electron donor.

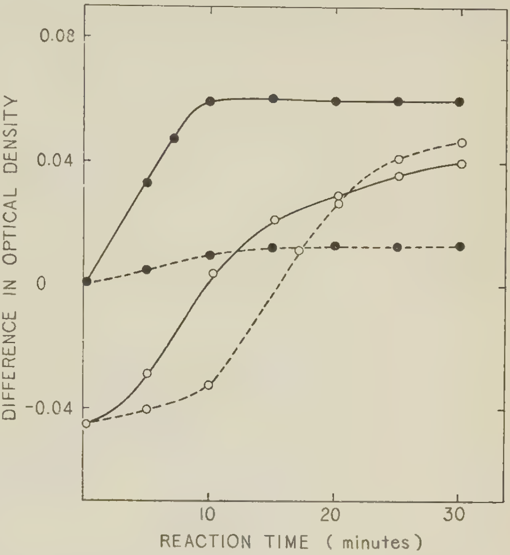

Fig. 3. Reduction of cytochrome b and a_2 in preparation with succinate.

—○— reduction of cytochrome b (OD_{561}-OD_{545}) —●— reduction of cytochrome a_2 (OD_{630}-OD_{650}) ---- in the presence of cyanide ($10^{-3}\,M$) —— in the absence of cyanide

Reaction was carried out at room temperature in photocell sealed with liquid parafin. Reaction was started by adding 0.2 ml. of $M/15$-succinate. Each photocell contained 1.5 ml. of soluble preparation and 1.3 ml. of $M/15$-phosphate buffer (pH 7.4). When cyanide was used, 0.3 ml. of cyanide ($10^{-2}\,M$) was added instead of the same volume of phosphate buffer. Measurement was carried out using Hitachi Photo-electric Spectrophotometer.

Effect of Cyanide on Oxidation of Reduced Cytochromes in Preparation—As shown in Fig. 4, the cytochrome b and a_2 once reduced by succinate, were oxidized in an instant by contacting with air. When the air was again removed from

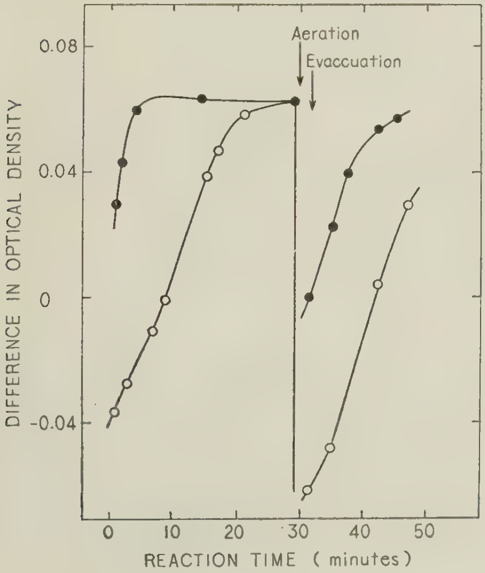

Fig. 4. Oxido-reduction of cytochrome b and a_2 in the preparation.

—○— oxido-reduction of cytochrome b (OD_{561}-OD_{545})

—●— oxido-reduction of cytochrome a_2 (OD_{630}-OD_{650})

Reaction was carried out at room temperature in Thunberg type photocell and before the reaction was started by the addition of $20\,\mu M$ of succinate, air was removed from the photocell.

Each photocell contained 1.5 ml. of the soluble preparation and 1.3 ml. of $M/15$-phosphate buffer (pH 7.4) in the main compartment and 0.2 ml. of $M/5$-succinate in the side arm. Measurement was carried out using Hitachi Photo-electric Spectrophotometer.

the cuvette, cytochromes again began to become their reduced forms. This oxido-reduction reaction could be repeated several times. On the other hand, the mode of oxidation of the reduced cytochromes was studied in the presence of cyanide. As shown in Fig. 5, in the presence of 10^{-3} *M* of cyanide, the oxidation of reduced cytochrome a_2 occurred only partially and the rate of the oxidation of cytochrome b was remarkably inhibited. On the other hand, in the presence of 10^{-2} *M* of cyanide, there was no detectable change in the oxido-reduction level of cytochrome a_2 and the rate of the oxidation of cytochrome b became very slow. These results strongly suggest that cytochrome b and a_2 were located between succinate (DPNH) and oxygen in the electron transport system and cytochrome b was located closer to succinate (DPNH) than cytochrome a_2.

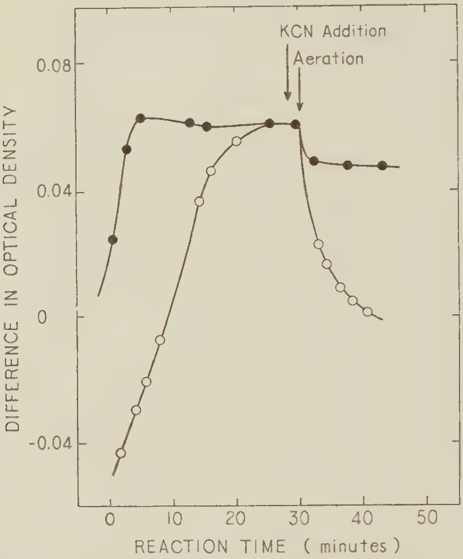

FIG. 5. Effect of cyanide on oxido-reduction of cytochrome b and a_2 in preparation.

—○— oxido-reduction of cytochrome b (OD_{561}-OD_{545})

—●— oxido-reduction of cytochrome a_2 (OD_{630}-OD_{650})

Reaction was carried out at room pemperature in Thunberg type photocell and before the reaction was started by the addition of 20 *μM* of succinate, air was removed from the photocell.

Each photocell contained 1.5 ml. of the soluble preparation, 1.0 ml. of *M*/15-phosphate buffer (pH 7.4) in the main compartment, 0.2 ml. of *M*/5-succinate in one side arm and 0.3 ml. of 10^{-2} *M* cyanide in the other side arm. Measurement was carried out using Hitachi Photoelectric Spectrophotometer.

Properties of Haemin of Cytochromes—Properties of haemin in the bacteria were studied spectrophotometrically. Fig. 6 showed the absorption spectrum of the haemins in ether solution. The ether solution to be measured was

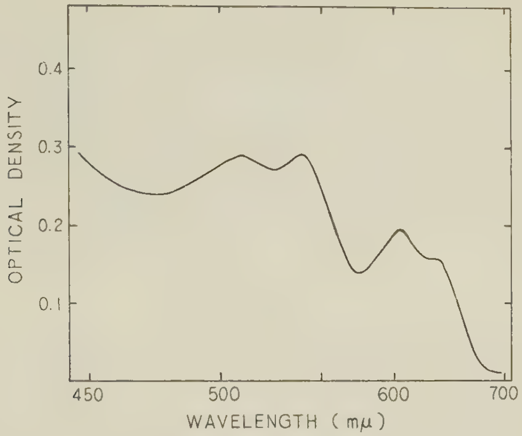

Fig. 6. Absorption spectrum of bacterial haemins in ethereal solution washed with 5% HCl.

Measurement was carried out using Beckman DK-2 Spectrophotometer.

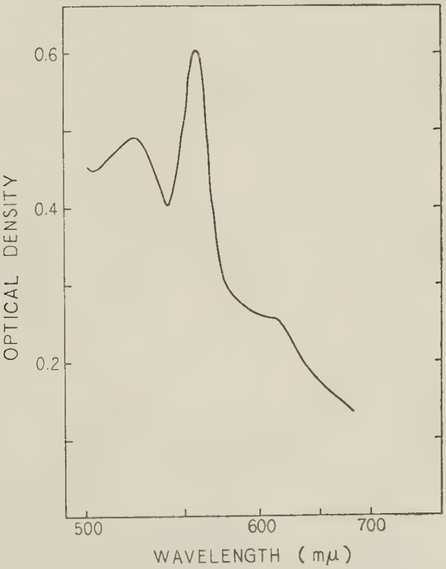

Fig. 7. Absorption spectrum of pyridine haemochromes.

Acetone solution of haemins dissolved in a mixture of pyridine and 0.2 *N* NaOH (1 : 2) was reduced by adding solid dithionite. Measurement was carried out using Backman DK-2 Spectrophotometer.

washed with 5 per cent HCl to ensure complete formation of the haemins. It showed a diffuse but strong band at 603 mμ in addition to the band of protohaemin at 635, 540 and 508 mμ. This band (603 mμ) showed a good agreement with that of cytochrome a_2 of Barrett's preparation (*5*) and the bands of protohaemin seemed to correspond to the haemin of cytochrome b_1. Fig. 7 showed the absorption spectrum of pyridine haemochrome. Pyridine haemochrome was formed by adding solid dithionite to haemins in a mixture of pyridine and 0.2 N NaOH (1 : 2). The ferrous compound gave a sharp peak at 557 mμ and a shoulder around 614 mμ. The peak at 557 mμ showed the existence of protohaemin and the shoulder around 614 mμ coincided with the peak corresponding to haemin of cytochrome a_2 of *Aerobacter aerogenes* (*5*).

DISCUSSION

Studies on the haem portion of cytochrome b and a_2 showed that cytochrome a_2 of this bacteria was similar to that found in *Aerobacter aerogenes* (*5*) and was different from that found in *Pseudomonus aeruginosa* (*7*). Cytochrome b of this bacteria was also similar to that found in *Escherichea coli* and *Aerobacter aerogenes* (*8*) which was called cytochrome b_1.

As described previously, any effort to separate the cytochromes in the preparation could not be successful. In addition, these cytochromes were not only precipitated by rather low speed ultracentrifugation but also easily salted out from the 20 per cent saturated solution of ammonium sulfate. These results strongly suggest that the cytochromes in the preparation existed as a multimolecular unit, namely as a kind of sub-unit of the particulate preparation. This suggestion may also be supported by the findings that the electron transport among cytochrome b_1, cytochrome a_2 and oxygen could be carried out in an instant. On the other hand, the slow reduction of cytochromes by succinate under anaerobic condition may be explained by a very low concentration of succinic dehydrogenase in the preparation. Therefore, in this preparation, the rate limiting step of the electron transfer between succinate and oxygen may be the step of succinate dehydrogenation.

The results shown in Figs. 4, 5 and 6 were summarized as follows.

a) Cyanide (10^{-3} M) strongly but not completely inhibited the oxido-reduction of cytochrome a_2.

b) Cyanide (10^{-3} M) did not inhibit the reduction of cytochrome b_1, whereas it inhibited the oxidation of cytochrome b_1 by oxygen. In addition, its oxidation was almost completely inhibited by 10^{-2} M concentration of cyanide, by which the action of cytochrome a_2 was almost completely inhibited.

From these facts, it may be concluded that the direction of the electron transport from either succinate or DPNH to oxygen in the preparation was shown in the following diagram.

$$\begin{tikzcd}
\text{DPNH—dehydrogenase} \arrow[dr, dash] & \\
& \text{cytochrome } b_1\text{—cytochrome } a_2\text{—oxygen} \\
\text{succinate—dehydrogenase} \arrow[ur, dash] &
\end{tikzcd}$$

It was also natural that the reduction of cytochrome b_1 always lagged behind that of cytochrome a_2, because the rate of electron transfer between cytochrome b_1 and a_2 was far more rapid than that between succinate or DPNH and cytochrome system, and the redox potential of the cytochrome a_2 might be higher than that of cytochrome b_1.

The terminal electron transport system of this bacteria seemed to be able to operate in the presence of 10^{-3} *M* concentration of cyanide, though the activity of cytochrome a_2 was remarkably inhibited by that concentration of cyanide. Therefore, the increase in the amount of cytochrome a_2 in this system may increase the electron transport capacity in the presence of 10^{-3} *M* concentration of cyanide. However, nothing could be studied on the physiological significance of cytochrome a_1 owing to its minute amount.

SUMMARY

1. Water soluble preparation which catalyzes the electron transfer between DPNH or succinate and oxygen was obtained from subcellular particulate preparation of a strain of *Achromobacter*.

2. Cytochrome b_1 and a_2 contained in the preparation were slowly reduced by succinate or DPNH and oxidized in an instant by oxygen.

3. Cyanide (10^{-3} *M*) remarkably but not completely inhibited the oxido-reduction of cytochrome a_2 but did not inhibit the reduction of cytochrome b_1.

4. The direction of electron transport between succinate or DPNH and oxygen was postulated as follows;

DPNH—dehydrogenase \
cytochrome b_1—cytochrome a_2—oxygen
succinate—dehydrogenase /

5. These results supported a view on the mechanism of cyanide resistance in this bacteria.

The authors wish to express their sincere appreciation to Dr. K. Sakaguchi for his encouragement and advice in this investigation, and are indebted to Dr. K. Okunuki Osaka University for his kind suggestion and discussion.

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ENZYMATIC FORMATION OF L-GULONOLACTONE FROM D-GLUCURONOLACTONE BY RAT LIVER MICROSOMES*

It has been established by several workers that the immediate precursor of L-ascorbic acid in animal tissue is L-gulonolactone (*1–8*). However the mechanism of formation of the lactone has not yet been elucidated completely. Recently, Yamada (*9*) demonstrated the enzymatic formation of L-gulonolactone from L-gulonate by the reverse reaction of lactonase I, and Mano *et al.* (*10*) have proved the direct conversion of D-glucuronolactone to L-gulonolactone by the action of TPN-gulonic dehydrogenase. In a recent report by Guha *et al.* (*11*) D-glucuronolactone is converted to L-ascorbic acid by goat liver microsomes in the presence of cyanide, but the formation of L-gulonolactone as intermediate in this conversion has not been established. The present paper, therefore, is concerned with the formation of L-gulonolactone by rat liver microsomes.

The liver from freshly killed rats was homogenized in 4 vol. of isotonic KCl. The resulting homogenate was centrifuged at 9,000×*g* for 15 minutes and the residue was discarded. The microsomes, isolated from this supernatant by centrifugation at 105,000×*g* for 60 minutes, were washed once with saline and resuspended in 0.1 *M* phosphate buffer, pH 7.0.

The reaction mixture, containing 60μmoles of D-glucuronolactone, 300μmoles of potassium cyanide, microsomes prepared from 0.8 g. of rat liver and 120 μmoles of phosphate buffer (pH 7.0) in a total volume of 6.0 ml., was incubated at 37° for 2 hours in an atmosphere of O_2 or N_2.

L-Gulonolactone was identified by paper chromatography, as follows: the reaction mixture was deproteinized with perchloric acid and filtered. The filtrate was neutralized with KOH, the precipitated potassium perchlorate was removed by filtration and after this filtrate was concentrated by lyophilization, it was spotted on Toyo Roshi No. 53 filter paper. In Table I are listed the R_f values in various solvents. The spots were developed by the following two methods: buffered neutral hydroxylamine reagent and a solution of $FeCl_3$ described by Eisenberg (*12*), and aqueous KIO_4 solution and borate–soluble starch reagent according to Metzenberg (*13*).

In the case of the incubation in N_2, L-gulonolactone was detected from the reaction mixture, as shown in Table I, while in O_2 it was not detected but the formation of ascorbic acid was observed as stated below. These findings suggest presumably that when the incubation was carried out in O_2, L-gulonolactone formed from D-glucuronolactone was converted immediately to ascorbic acid by the action of L-gulonolactone dehydrogenase present in microsomes, while in the anaerobic incubation the lactone might be unchanged and accumulated.

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* This work has been published in Japaneses. (*J. Japan. Biochem. Soc.*, **31**, 708, 1959)

TABLE I

Identification of L-Gulonolactone on Paper Chromatography

Solvent used	R_f Butanol-ethanol-water (4 : 1 : 1)	Acetone-water (4 : 1)	Ethanol-acetic acid-water (8:1:1)
Authentic sample			
D-Glucuronolactone	0.34	0.83	0.61
L-Gulonolactone	0.24	0.71	0.50
Reaction product in N_2 1)	0.34	0.83	0.61
	0.24	0.70	0.50
	0.00	0.14	0.27
		0.07	0.17
Reaction product in O_2 2)	0.34	0.83	0.61
	0.10	0.15	0.27
	0.05	0.07	0.17
Reaction product without cyanide	0.34	0.84	0.60
	0.10		

1) The reaction conditions are indicated in the text.

2) The spot corresponding to ascorbic acid could be scarcely observed by the reagent of Metzenberg.

Ascorbic acid formed in aerobic incubation was identified by paper chromatography of the trichloroacetic acid filtrate of the reaction mixture according to Mapson (*14*). The filtrate was treated with 2,4-dinitrophenylhydrazine after oxidation with bromine. For comparison, 2,4-dinitrophenylhydrazone of 2,3-diketo-L-gulonic acid was prepared from ascorbic acid, after oxidation with bromine, in a similar manner. The hydrazones were analysed by paper chromatography with an aqueous solvent containing 10 per cent (*v/v*) acetic acid, 10 per cent (*v/v*) glycerol and 10 per cent (*w/v*) phenol; both hydrazones showed an R_f value of 0.68. By using the upper phase of a mixture composed of *tert*-amylalcohol-ethanol-water (50 : 5 : 40 by vol.), the hydrazones ran with an R_f of 0.62. These spots of the hydrazone derived from ascorbic acid were not detectable either when cyanide was absent in the reaction mixture, or the reaction was carried out in the atmosphere of N_2.

In order to examine the specificity of the microsomal enzyme for substrate, various uronic acids and onic acids were tested, and the formed ascorbic acid was determined by the method of Roe and Kuether (*15*). The results are shown in Table II. The enzyme was active only on D-glucuronolactone, ethyl D-glucuronate, L-gulonolactone, and L-galactonolactone.

In summary, it was proved that the microsomes of rat liver, in the presence of cyanide under the anaerobic condition, are able to convert D-glucuronolactone to L-gulonolactone which in turn is converted under the aerobic condition to ascorbic acid promptly. The presence of cyanide was essential for the formation of L-gulonolactone from D-glucuronolactone but the role of cyanide has not been clarified at present.

TABLE II

Substrate Specificity of the Microsomal Enzyme[1]

Substrate	Ascorbic acid formed (μg.)	
D-Glucuronolactone	154	(0)[2]
D-Glucuronate	0	
Ethyl D-glucuronate	94	(0)[2]
D-Galacturonate	0	
D-Manuronolactone	0	
D-Manuronate	0	
L-Gulonolactone	90	(179)[2]
L-Gulonate	0	
D-Gulonolactone	0	
D-Gulonate	0	
L-Galactonolactone	225	(133)[2]
L-Galactonate	0	
D-Galoctonolactone	0	
D-Galactonate	0	

1) The assay conditions were as follows: to 10 μmoles of each of the above substrates were added microsomes prepared from 0.4 g. of rat liver, 150 μmoles of potassium cyanide and 60 μmoles of phosphate buffer, pH 7.0, in a final volume 3.0 ml. The incubation was carried out in an atmosphere of O_2 for 2 hours at 37°, and the ascorbic acid formed was determined after the reaction was stopped by the addition of 20% trichloroacetic acid.

2) Figures in the parentheses indicate the values of ascorbic acid formed when cyanide is omitted from the reaction mixture.

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